

# **The role of Vav proteins in macrophage morphology and migration**

by

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all my teachers and  
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## **Abstract**

The Rho family GTPases are key signalling components that regulate the cytoskeleton and adhesion during cell migration. The Vav family of proteins act as guanine nucleotide exchange factors for several Rho GTPases. There are three isoforms of Vav expressed in mammalian cells: Vav1, the expression of which is largely restricted to haematopoietic cells, Vav2 and Vav3. In this study the role of Vav proteins in macrophage migration has been investigated using macrophages derived from mice lacking one or all three Vav isoforms.

Cell migration and morphology were not significantly affected when single isoforms of Vav were absent from macrophages. However macrophages lacking all three isoforms adopted an elongated morphology in culture, which resulted in more persistent cell migration. Vav proteins were not required for chemotaxis to the macrophage chemo-attractant, colony-stimulating factor-1 (CSF-1). Vav proteins were also not required for CSF-1-stimulated Rac1 activation or Rac-mediated cytoskeletal reorganization in response to CSF-1. However, in response to CSF-1 stimulation Vav1 and Vav3 were phosphorylated on tyrosine residues, which has previously been shown to regulate their GEF catalytic activity. Macrophages lacking all three Vav proteins were defective in spreading upon adhesion to both glass and plastic. The defect correlated with reduced activation of Rac1 and RhoA, and a reduction in the activation of Erk1/2 and phosphorylation of paxillin in response to adhesion.

Vav proteins are therefore not required for directed macrophage migration to the chemo-attractant CSF-1, but have an important role in adhesion-dependent signalling and are needed to maintain normal macrophage migration and morphology.

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### **Abbreviations**

ACE	Angiotensin-converting enzyme
ADF	actin depolymerizing factor
ADP	adenosine diphosphate
APC	Adenomatous Polyposis coli
Arp	actin related protein
ATP	adenosine triphosphate
BMM	bone marrow-derived macrophages
BRC	B cell receptor
BSA	bovine serum albumin
CH	Calponin homology domain
CLIP170	cytoplasmic linker protein of 170 kDa
CSF-1	colony-stimulating factor-1
CSF-1R	colony-stimulating factor -1 receptor
DH	Dbl homology domain
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediamine tetra-acetic acid
EGF	epidermal growth factor
Ena/VASP	(Drosophila) enabled/vasodilator-stimulator protein
ERK	extracellular signal-regulated kinase

FACS	fluorescence-activated cell sorting
FAK	focal adhesion kinase
Fc $\gamma$ R	Fc-gamma chain receptor
FCS	foetal calf serum
fMLP	N-formyl-methionyl-leucyl-phenylalanine
GDI	guanine nucleotide dissociation inhibitor
GDP	guanosine diphosphate
GIT1	G protein-coupled receptor kinase interactor 1
Grb2	growth factor receptor-bound protein 2
GST	glutathione S-transferase
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
HSPC300	haematopoietic stem cell progenitor 300
ICAM-1	intercellular adhesion molecule 1
IL	interleukin
IPTG	isopropyl- $\beta$ -D-thiogalactopyranoside
IQGAP1	IQ motif-containing GTPase activating protein-1
IRS-53	insulin receptor substrate of 53 kDa
LB	Luria-Bertani
LFA-1	leukocyte function-associated antigen
LIMK	LIM domain kinase
LPA	lysophosphatidic acid
MCP-1	monocyte chemoattractant peptide-1
mDia	mammalian homolog of <i>Drosophila</i> diaphanous
MEK	MAPK-ERK kinase
MES	2-(N-morpholino)ethane sulphonic acid
MIDAS	metal ion-dependent adhesion site
MLC	myosin light chain

MMP	matric metalloproteinase
MOPS	3-(N-morpholino)propane sulphonic acid
NADPH	nicotinamide adenine dinucleotide phosphate
Nap	Nck-associated protein
NFAT	nuclear factor of activated T cells
Np-40	nonidet P-40
Op18	oncoprotein 18
p130CAS	p130 Crk-associated substrate
PAK	p21-activated kinase
PBS	phosphate-buffered saline
PH	Pleckstrin homology
PI3K	phosphoinositol 3-kinase
PIP	phosphatidyl inositol phosphate
PIR121	p53-inducible mRNA-121
PIX	PAK interacting exchange factor
PKA	cAMP dependent protein kinase
PKC	protein kinase C
PKG	cGMP dependent protein kinase
PLC- $\gamma$ 1	phospholipase C- $\gamma$ 1
PTEN	phosphatase and tensin homolog
PTPN22	protein tyrosine phosphatase non-receptor type 22
Pyk2	proline-rich tyrosine kinase 2
RANTES	regulated upon activation, normal T cell expressed and secreted
RNA	ribonucleic acid
ROCK	Rho kinase
RPMI	Roswell Park Memorial Institute
RTK	receptor tyrosine kinase
s.e.m.	standard error of the mean

Scar	Suppressor of cAR
SDF-1	stromal cell-derived factor-1 (CXCL12)
SDS	sodium dodecylsulphate
SH2	Src homology domain 2
SH3	Src homology domain 3
SHIP	SH2 domain-containing inositol phosphatase
SHP-1	haemopoietic cell protein tyrosine phosphatase-1
siRNA	small interfering ribonucleic acid
SLP-76	SH2 domain-containing leukocyte protein of 76 kDa
TBS	Tris-buffered saline
TCR	T cell receptor
Temed	N,N,N',N'-tetramethyl-ethylene-diamine
TRIS	Tris(hydroxymethyl)-aminomethane
TRITC	tetramethyl-rhodamine-isothiocyanate
VCAM-1	vascular cell adhesion molecule-1
WASp	Wiskott-Aldrich syndrome protein
WCL	whole cell lysate
WIP	WASp interacting protein
ZAP70	zeta chain-associated protein kinase of 70 kDa
ZF	zinc-finger domain

#### **List of movies on DVD-ROM**

movie	BMM genotype	Figure
movie 1	Wt	3.2
movie 2	Vav1 <sup>-/-</sup>	
movie 3	Vav2 <sup>-/-</sup>	
movie 4	Vav3 <sup>-/-</sup>	

movie 5	Wt	3.3
movie 6	Wt	3.6
movie 7	Vav1/2/3 <sup>-/-</sup>	
movie 8	Wt BMM tissue culture plastic	3.8
movie 9	Wt BMM bacterial plastic	
movie 10	Wt BMM	3.9
movie 11	Vav1/2/3 <sup>-/-</sup>	
movie 12	Wt	4.13
movie 13	Vav1/2/3 <sup>-/-</sup>	
movie 14	Wt	5.2
movie 15	Vav1/2/3 <sup>-/-</sup>	
movie 16	Wt	5.4
movie 17	Vav1 <sup>-/-</sup>	
movie 18	Vav2 <sup>-/-</sup>	
movie 19	Vav3 <sup>-/-</sup>	

## **1. Introduction**

### **1.1 The actin cytoskeleton and microtubule network in migration**

Cell migration is an essential biological process in normal development and tissue homeostasis, but also in disease states such as cancer and atherosclerosis. Migration is a product of coordinated regulation between changes in cell morphology and adhesion. Cell morphology is determined by organisation of the cytoskeleton. The cytoskeleton comprises of filamentous structures formed by the polymerisation of different proteins. These include actin filaments (microfilaments), microtubules, intermediate filaments and septins.

#### **1.1.1 Actin**

Actin filaments are composed of a double-helical arrangement of a pair of actin polymers. The filament has a polarity that is conferred upon it by the inherent polarity of the actin monomers and their head-to-tail arrangement in the filament. Monomeric actin has different association kinetics at the two ends of the filament. At the 'barbed' end, actin has a greater tendency to bind the polymer, whereas at the 'pointed' end, its dissociation is relatively greater (Pollard 1986).

Free actin monomers are ATP-bound. Once polymerised into the actin filament, these proteins hydrolyse the  $\gamma$ -phosphate group of the bound ATP molecule in a time-dependent manner. This event is associated with filament destabilisation. Spontaneous polymerisation of actin monomers is an energetically unfavourable reaction, however once a filament has been nucleated the rate of polymerisation is considerably faster (Pollard, 1986), and limited only by the concentration of available actin monomers. It is thought that in the cell, actin-ATP is maintained at a much higher concentration than that which is limiting to polymerisation (Pollard and Cooper, 1984), and therefore new filament nucleation is the rate-limiting step of actin polymerisation (Pollard, 2000).



Actin filaments are quite flexible, and it is unlikely that individual filaments can generate enough force to effect cell protrusion. However, electron microscopy of the lamellipodium of migrating fish keratocytes showed how actin filaments could induce membrane protrusion (Svitkina et al., 1997). It was observed that the actin at the actively protruding edge was organised into a dense meshwork of extensively branched filaments (Figure 1.1). In contrast, further away from the protruding edge, actin filaments were longer and un-branched. More recently, it was shown that lamellipodium formation induced by EGF in the metastatic MTLn3 cell line coincides with an increase in the number of barbed ends, where newly polymerised actin is more prominent (Chan et al., 2000). Therefore, though actin filaments themselves are flexible, when organised into a dense meshwork of branched filaments, the force generated by polymerisation is increased and may be sufficient for cell protrusion. Recent studies suggest that the force generated by actin polymerisation is sufficient to cause deformation of the plasma membrane (Mogilner and Oster, 2003), and exceed that required for lamellipodium protrusion (Prass et al., 2006).



**Figure 1.1: Actin filaments in lamellipodia.** The figure shows electron micrographs of two regions of the lamellipodium of a migrating fish keratocyte. The left panel shows the filament structure at the leading edge, which contains a dense meshwork of extensively branched filaments. The right panel is a section of the middle part of the same lamellipodium, which has longer filaments with less branching. Taken from Svitkina et al., 1997.

#### 1.1.1.1 Regulatory components of actin filament nucleation

Actin filaments in the extreme edge of the protruding lamellipodia are extensively branched, therefore new filament nucleation correlates with actin filament branching. The Arp2/3 protein complex was shown to localise at the branch-points of actin filament arrays (Svitkina and Borisy, 1999). This complex consists of the two actin-related proteins Arp2 and Arp3, with five other proteins (ARPC1-5) (Robinson et al., 2001). The Arp2/3 complex catalyses the nucleation of actin filaments on the side of existing ones at an angle of 70° (Robinson et al., 2001). Activation of the Arp2/3 complex thereby promotes actin polymerisation.

Activation of the Arp2/3 complex is mediated by its interaction with nucleation promoting factors such as the Wiskott-Aldrich Syndrome protein (WASp) and Scar/WAVE proteins (Macheski and Insall, 1998). WASp adopts an auto-inhibitory conformation in the absence of the appropriate stimuli. This conformation conceals a GTPase-binding domain as well as a portion of the protein that interacts with the Arp2/3 complex. However, the active Rho family GTPase Cdc42 (section 1.2) and the second messenger PIP<sub>2</sub> relieve this inhibition and induce an open conformation (Ma et al., 1998; Higgs and Pollard, 2000).

In vitro studies suggest a similar mechanism of regulation of WAVE proteins, however, Rac does not interact directly with WAVE. In vitro, WAVE activity is inhibited when it forms a complex with associated proteins PIR121, Nap25 and HSPC300. Upon interaction with active Rac1 and the adapter protein Nck, the inhibition of WAVE is relieved, possibly by dissociation of the protein complex (Eden et al., 2002). In a cellular context it appears that WAVE remains in complex with these proteins independent of Rac1 activation, but that Rac1 activity causes a relocalisation of the WAVE complex to the leading edge of the cell, promoting cell protrusion (Steffan et al., 2004). The insulin receptor substrate-53 (IRS-53) has also been reported to bind WAVE (Miki et al., 2000). Rac associates with IRS-53, forming a trimolecular complex that is

able to induce membrane ruffling (Miki et al., 2000). Interestingly, IRS-53 has also been identified as a binding partner for Cdc42 (Govind et al., 2001).

The rate of actin polymerisation is dependent on filament nucleation and the concentration free barbed ends. To control actin polymerisation the barbed ends can be 'capped' by the binding of capping proteins such as CapZ. The Ena/VASP family of proteins have also been implicated in the direct regulation of filamentous actin. However, rather than nucleating new actin filaments, the Ena/VASP proteins promote actin polymerisation by inhibiting barbed end capping. This function for Ena/VASP was suggested by Bear and colleagues, who showed that sequestration of Ena/VASP to intracellular compartments led to increased branching of actin filaments in newly formed protrusions, whereas constitutive membrane localisation led to elongated actin filaments (Bear et al., 2002).

The Formins are another family of proteins that can nucleate actin filaments (Evangelista et al., 2003). Whereas the Arp2/3 complex nucleates new filaments by creating branchpoints on existing ones, formins can generate new actin filaments by stabilising dimers of actin and thus creating barbed ends. The actin nucleating activity of the Diaphanous sub-family of formins is suppressed by an auto-inhibitory sequence at the N-terminus, and can be activated by RhoA binding to a Rho-binding domain at the C-terminus (Li and Higgs, 2003).

#### 1.1.1.2 Actin polymerisation and cell protrusion

The culmination of signalling from the plasma membrane to regulate actin nucleation via the Arp2/3 complex forms the basis for the current model for actin polymerisation at the leading edge of migratory cells, called the 'dendritic nucleation/array treadmilling hypothesis' (described in detail in (Pollard et al., 2003)). Free ATP-bound actin monomers are associated with profilin. Activation of the WASp/WAVE proteins by upstream signalling elements such as PIP<sub>2</sub> or Rho GTPases (described in section 1.2) results in the activation of the Arp2/3 complex. This protein complex binds actin monomers and creates

new filaments by initiating branches off existing filaments. As more actin monomers are added to the newly created filaments, the actin subunits in the more mature parts of the filament hydrolyse the bound ATP to ADP. This attracts the protein ADF/cofilin, which promotes severing and depolymerisation of filaments. ADP-actin monomers released by the action of ADF/cofilin are then bound by profilin, which promotes the exchange of ADP for ATP, replenishing the pool of free actin. New filament length is controlled by capping proteins, which prevents further addition to the filament, as well as the severing activity of ADF/cofilin. New filament nucleation and polymerisation at the front of the filament array, and filament destabilisation of mature filaments leads to an overall 'treadmilling' of the actin network. This is therefore a means by which forward force is generated to create membrane protrusion.

#### 1.1.1.3 Generation of different actin structures

Cells are able to construct different types of cell protrusions such as broad, sheet-like lamellipodia, and the finger-like filopodia. These structures are thought to have specific roles in migration (reviewed in (Rorth, 2003)). For example, lamellipodia may be required during migration to provide enough traction to pull the rest of the cell forward. The relatively finer filopodia, in contrast, may be required for sensing of the cellular environment before the cell commits to migration. Extracellular stimuli to which the cell is exposed may determine which structure is produced. However, it appears that a variety of stimuli and signal transduction pathways signal to a relatively small number of components that regulate the organisation of the actin cytoskeleton. Differences in the interactions and localisation of these components may provide the mechanism to generate distinct filamentous actin structures.

Different organisations of actin filaments can be generated by the actin filament nucleating factor. As described above, the Arp2/3 complex produces branched networks of actin filaments that are observed in lamellipodia. In contrast, the formin mDia1 generates bundles of actin filaments that are

associated with filopodia (Li and Higgs, 2003), and Ena/VASP promotes the formation of long un-branched filaments (Bear et al., 2002). In addition, different Arp2/3 activators may promote different types of F-actin organisations. The WAVE protein is localised in newly formed lamellipodia of mouse melanoma cells but not in filopodia. The latter preferentially recruited Ena/VASP (Hahne et al., 2001). The Ena/VASP proteins have also been shown to localise in focal adhesions associated with stress fibres, which are bundled actin filaments (Gertler et al., 1996). Diversity in F-actin organisation may also be brought about by accessory proteins, which interact with the basic components regulating actin polymerisation. For example, in migrating *Dictyostelium* the WASp-interacting protein (WIP) specifically promoted the formation of filopodia at the extreme edge of the pseudopod (Myers et al., 2006). Indeed, it has been demonstrated that WASp and WAVE proteins activate actin nucleation via Arp2/3 complex at significantly different rates *in vitro* (Zalevsky et al., 2001), which could also contribute to the generation of different F-actin structures.

### 1.1.2 Microtubules

Microtubules are composed of a cylindrical arrangement of 13 protofilaments of  $\alpha$  and  $\beta$  tubulin dimers. The particular orientation of the tubulin dimers confers a polarity upon the filament, creating 'plus' and 'minus' ends. Microtubules undergo a process called dynamic instability, where the filament 'plus' end switches between states of rapid depolymerisation (catastrophe) and polymerisation (rescue). Both subunits bind GTP, but  $\beta$ -tubulin can hydrolyse bound GTP and can therefore exist in GDP- and GTP-bound conformations. The GTP-bound conformation of the tubulin dimer is thought to be required for addition to the polymer during the growth phase. The bound GTP is hydrolysed over time, causing tubulin dissociation during catastrophe (reviewed in (Inoué and Salmon, 1995)).

### 1.1.2.1 Microtubules in cell protrusion

Whereas the filamentous actin network is generally accepted as the major means of force generation during migration, the contribution of microtubules to migration appears to depend on cell type. In neutrophils, microtubule disruption causes an increase in cell migration (Keller et al., 1984), whereas it is detrimental to the migration of fibroblasts (Bershadsky et al., 1991). However, in macrophages microtubule disruption causes a loss of cell polarity and directed migration (Glasgow and Danielle, 1994). Therefore in macrophages, microtubules may not be essential for cell protrusion, but may play an important role in directed and persistent cell migration.

It has been reported that microtubules could effect cell protrusion in themselves by exerting a protrusive force at their plus ends (Howard and Hyman, 2003). Alternatively, microtubules may also promote protrusion by affecting the regulation of the actin cytoskeleton. Induction of tubulin polymerisation at the leading edge was shown to induce Rac activation, protrusion of lamellipodia and membrane ruffling (Waterman-Storer et al., 1999).

It has also been proposed that vesicle trafficking to, and secretion at the leading edge of migrating cells provides cell membrane and other components required for continued cell protrusion and new adhesion formation (reviewed in (Nabi, 1999)). Preferential delivery of secretory vesicles towards the leading edge of migrating cells has been demonstrated in fibroblasts (Hopkins et al., 1994). Vesicle transport along microtubules towards their 'plus' end is mediated by motor proteins that combine ATPase activity and microtubule binding to transport vesicles. Kinesins are a subfamily of motor proteins. The role of vesicular trafficking in polarity was demonstrated by Rodionov and colleagues. Antibodies raised against the motor domain of kinesin disrupted the polarised shape of fibroblasts in a similar way to the microtubule-destabilising drug nocodazole (Rodionov et al., 1993).

### 1.1.2.2 Microtubules in cell adhesion

A number of studies indicate that microtubules have an important role in regulating cell adhesion during migration. Adhesions are regulated in a polarised manner during migration, being formed at the leading edge, and disassembled from the tail during retraction. In melanoma cells it was shown that whereas microtubules were not required for cell protrusion, disruption of microtubules inhibited migration. This coincided with the development of long tails behind the advancing cell body (Ballestrem et al., 2000). In fish fibroblasts, microtubule ends were shown to target adhesion complexes repeatedly (Kaverina et al., 1998, Krylyshkina et al., 2003). This interaction was followed by the disassembly of the adhesion complex (Kaverina et al., 1999). It has been suggested that preferential targeting of adhesions could be a means by which microtubules can establish or maintain cell polarity.

Regardless of their specific roles in migration, the microtubule network of migrating cells has a distinct polarisation (Nabi, 1999). A greater proportion of the microtubules that are extended in the direction of the protruding front of the cell are stabilised (Gundersen and Bulinski, 1988) and in migrating cells, the centrosome is usually oriented towards the front of the cell (Wittman and Waterman-Storer, 2001). Furthermore, microtubules have been observed to preferentially grow into protrusions at the front of the cell (Waterman-Storer and Salmon, 1997). It is clear that microtubules can contribute to cell migration in a number of ways, though the extent of this is dependent on the cell-type. In all cases however, the inherent polarity of the microtubule network appears to be the basis of its contribution.

### 1.2 Rho GTPases and Migration

Rho GTPases are molecular switches that exist in GTP-bound (active) or GDP-bound (inactive) states, and can thereby be regulated to effect numerous cellular responses to extracellular stimuli. Accumulated evidence has shown Rho GTPases to be critical regulators of the microtubule and actin

cytoskeleton and adhesion. The Rho GTPases are therefore essential for cell migration.

### 1.2.1 The Rho family of small GTPases

In humans there are at least 23 different proteins identified as being part of the Ras-like, Rho family of small GTPases. These proteins can be subdivided into six subfamilies; Rho, Cdc42, Rac, RhoBTB, Rnd and RhoT (reviewed in (Bustello et al., 2007)).



**Figure 1.2: The Rho Family of small GTPases.** From Bustelo et al., 2007

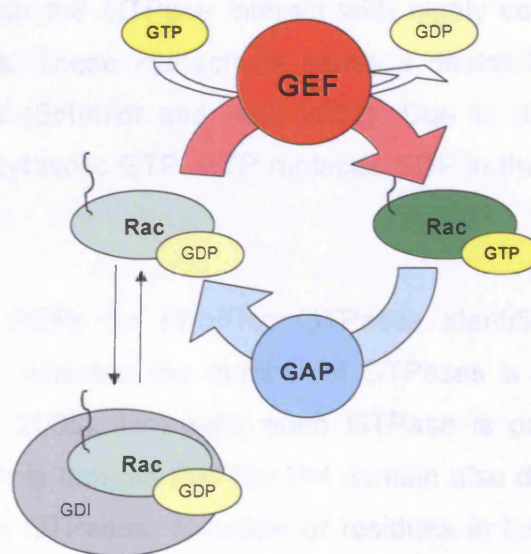
The Rho family members are guanine nucleotide binding proteins that through their inherent catalytic activity are able to exist in GTP-bound and GDP-bound conformations. In the GDP-bound conformation, they have much lower affinity for their effectors. Upon binding GTP, a conformation is induced that increases their interaction with their effector proteins. Upstream elements regulate Rho GTPase signalling by control of the transition between inactive and active states. Thus, they are able to act as molecular switches for numerous cellular processes (see reviews (Van-Aelst and D'souza-Shorey, 1997; Etienne-Manneville and Hall, 2002)).



### 1.2.2.1 Guanine Nucleotide Exchange Factors

### 1.2.2 Regulation of the Rho GTPases

GEF proteins all contain a domain homologous to a 233 amino acid (aa) Rho GTPase activation from the GDP-bound to GTP-bound state is catalysed by guanine nucleotide exchange factors (GEFs), (reviewed in (Rossman et al., 2005; Schmidt and Hall, 2002)). Rho GTPases have intrinsic catalytic activity that hydrolyses bound GTP to GDP. This activity is enhanced by the GTPase activating proteins (GAPs), which increase the rate of this reaction, thereby inactivating the GTPase. In addition, the spontaneous acquisition of free GTP by the GTPases is prevented by guanine nucleotide dissociation inhibitors (GDIs). These proteins preferentially associate with the GDP-bound GTPase, preventing exchange of the bound GDP for free GTP, and sequester the GTPase away from the membrane (Vav Aelst and D'souza-Shorey, 1997).



**Figure 1.3: Rho family small GTPase regulation.** Rho family small GTPases such as Rac are regulated by activating GEFs and inactivating GAPs. GDIs retain GTPases in their inactive state and away from their membrane localisation.

### 1.2.2.1 Guanine Nucleotide Exchange Factors

GEF proteins all contain a domain homologous to a 238 amino acid (aa) sequence in the Dbl protein (Rossman et al., 2005). Dbl was initially identified as an activator of the Rho GTPase Cdc42 in vitro. Following this it was shown that a minimum sequence of 238 aa of Dbl were required to catalyse the displacement of bound GDP from Cdc42 in vitro (Hart et al., 1994). With some exceptions (Meller et al, 2005), GEFs of the Rho/Rac family GTPases catalyse guanine nucleotide exchange via domains homologous to the minimum catalytic domain of Dbl, called the Dbl-homology (DH) domain (Rossman et al., 2005). Crystal structures comparing the free and GTPase-bound forms of GEFs show that there are two helices that protrude from the protein and interact directly with the GTPase (Worthylake et al., 2004). These helices interact with the guanine nucleotide binding region of the GTPase (Boriack-Sjodin et al., 1998). Conformationally variable regions (called Switch I and Switch II) on the GTPase interact with highly conserved residues in these two helices. These interactions cause a destabilisation of the GDP-GTPase complex (Schmidt and Hall, 2002). Due to the highly favourable association with cytosolic GTP, GTP replaces GDP in the GTPase nucleotide binding site.

The number of GEFs for Rho/Rac GTPases identified to date number approximately 60, whereas the number of GTPases is about a third of this (Rossman et al., 2005). However, each GTPase is only recognised by a subset of GEFs. It is thought that the DH domain also defines the specificity of interaction with GTPases. Mutation of residues in the DH domain of the Cdc42/RhoA GEF Dbs were used to alter its specificity towards different GTPases (Cheng et al., 2002). Interestingly, the PH domain that is invariably coupled to the DH domain may also contribute to substrate specificity. However, the role appears to differ according to the GEF. The GTP exchange on Rac1 by P-Rex2 was shown to depend on regions in the PH domain (Joseph and Norris, 2005). However, specific recognition of RhoA by PDZ-RhoGEF is independent of its PH domain (Oleksy et al., 2006).

#### 1.2.2.2 Rho GTPase Regulation by localisation, phosphorylation and expression

As well as the regulation of their GDP/GTP-bound states, the Rho GTPases can also be regulated by their localisation. Members of the Rho family GTPases are post-translationally modified at their C-terminus (with exceptions, for example RhoBTB (Rivero et al., 2001)). A CAAX (Cys-aliphatic-aliphatic-any aa) box sequence directs the addition of geranyl-geranyl or farnesyl groups. This modification is catalysed by geranyl-geranyl or farnesyl transferases respectively. After the addition of the lipid group the A-A-X moiety is cleaved and the C-terminal cysteine is carboxymethylated. The presence of the hydrophilic group enables membrane localisation of the GTPases (Takai et al., 2001). The nature of this modification can result in localisation to different cellular compartments. For example, RhoB is both geranyl-geranylated and farnesylated and localises to the plasma membrane. But when the farnesylation is inhibited, RhoB is targeted to multivesicular late endosomes (Wherlock et al., 2004). In addition, the regions proximal to the CAAX box may also have an important influence on localisation. For example, RhoG has a mainly vesicular localisation in COS cells, whereas Rac1 is localised in the cell membrane. Substitution of RhoG C-terminal sequences with that of Rac1 confer a membrane localisation upon RhoG (Prieto-Sanchez and Bustelo, 2003).

There is also evidence to suggest that Rho proteins can be regulated by phosphorylation and alteration of their expression levels. In NIH3T3 cells the E3 ubiquitin ligase Smurf1 was shown to cause the down-regulation of RhoA expression (Wang et al., 2003). Signalling through RhoA is also affected by direct phosphorylation by PKA (Lang et al., 1996) and PKG (Sawada et al., 2001). Phospho-mimicking mutants of RhoA were used to demonstrate that phosphorylation could potentially inhibit RhoA signalling in cells (Ellerbroek et al., 2003). In vitro enhancement of the phosphorylation state of Cdc42 and RhoA increased their association with RhoGDI (Forget et al., 2002). And in smooth muscle cells the phosphorylation of RhoA enhanced its association with RhoGDI and increased its stability (Rolli-Derkinderen et al., 2005). The

Rnd sub-family of Rho GTPases have undetectable levels of intrinsic catalytic activity and therefore cannot be regulated as other GTPases. The function of Rnd3/RhoE is regulated by phosphorylation, which changes its stability (Riento et al., 2005). In addition, RhoE is regulated transcriptionally by p53 in response to DNA damage (Onguasaha et al., 2006). The upregulation of RhoB mRNA and protein levels by the synthetic glucocorticoid Dex has also been reported (Chen et al., 2006).

### 1.2.3 Rho GTPase interaction with effectors

Once activated the Rho GTPases interact with their effector proteins, thus converting upstream signals into cellular responses. Study of Rho GTPase crystal structure when bound to GTP or non-hydrolysable GTP analogues identified the regions of the proteins that undergo major conformational changes upon GTPase activation. These include the switch I region (also known as the effector domain) and switch II region (Ihara et al., 1998). Thus, effector proteins specifically recognise and interact with the GTP-bound conformation of the effector domain. Different effectors of the same GTPases recognise distinct regions within the effector domain, explaining to some extent how single GTPases can induce multiple cellular processes. By mutating specific residues in the effector region it was shown that different mutations could prevent the association of a subset of effectors (Lamarche et al., 1996; Sahai et al., 1998). However in many cases certain sequences outside the effector region contribute to binding specificity (Bishop and Hall, 2000).

Many of the effectors of the Rho GTPases have a common mechanism of activation by GTP-bound GTPase (reviewed in (Bishop and Hall, 2000)). These effectors exist in an auto-inhibited state that is relieved by interaction with the GTPase. For example, the Rac and Cdc42 effector p21-activated kinase-1 (PAK1) contains an auto-inhibitory domain that binds and inhibits the catalytic domain of this serine/threonine kinase. It was shown that Cdc42 is capable of binding the auto-inhibitory domain, and thus relieves the inhibition of the catalytic activity of PAK1 (Tu and Wigler, 1999). Another example is the

formin protein Dia, which upon binding Rho catalyses the polymerisation of actin. The 389 N-terminal residues of Dia inhibit its activity. Active RhoA binds to this region and relieves the inhibition (Li and Higgs, 2003).

#### 1.2.4 Roles of Rho GTPases in migration

The polymerisation of actin can be regulated in different ways to produce morphological changes during migration. It is now widely accepted that the regulation of the actin cytoskeleton is mediated by the Rho family of GTPases, of which the Rho, Rac and Cdc42 subfamilies are the most extensively studied and characterised (Jaffe and Hall, 2005). Studies show that Rho GTPases also contribute to migration by affecting cell polarity.

##### 1.2.4.1 Rho GTPases in protrusion and retraction

The ability of the Rho GTPase family members to remodel the actin cytoskeleton in distinctly different ways is a means by which cell motility can be achieved. Rho has been shown to regulate the formation of actin stress fibres and focal adhesions in fibroblasts (Ridley and Hall, 1992), whereas Rac induces membrane protrusion and ruffles (Ridley et al., 1992), and Cdc42 promotes the formation of finger-like protrusions called filopodia (Kozma et al., 1995). Studies of the role of RhoA, Cdc42 and Rac in migratory cells such as macrophages demonstrate that their regulation of the actin cytoskeleton could affect both cell protrusion and contraction during migration (Figure 1.4). Active Rac1 mutants induced lamellipodia and membrane ruffles in macrophages, whereas active Cdc42 induced filopodia (Allen et al., 1997). RhoA induced cell contraction, whereas RhoA inhibition led to the development of long trailing tails.

**Figure 1.4: Regulation of the actin cytoskeleton in macrophages by Rac, Cdc42 and RhoA.** Expression of constitutively active mutants of Rho GTPases in macrophages has distinct effects on the actin cytoskeleton. Rac1 induces membrane ruffles and lamellipodia (A). Cdc42 induces formation of filopodia (B), and RhoA induced cell-rounding (C). Taken from Allen et al., 1997.

Further studies showed how these morphological changes related to cell migration. Cdc42 was required for directionally persistent migration of macrophages (Allen et al., 1998) and neutrophils (Szczur et al., 2006). It is generally thought that Rac promotes the formation of lamellipodia and ruffles at the leading edge of migrating cells (Ridley et al., 2003). Expression of dominant negative Rac1 in macrophage cell lines significantly reduces migration speed (Allen et al., 1998), where dominant negative Rac2 prevents membrane ruffling (Abell et al., 2004). Rac1- and Rac1/2-deficient macrophages have an elongated morphology in culture (Wheeler et al., 2006), and Rac-1 deficient macrophages have defects cell spreading and membrane ruffling (Wells et al., 2004). Rac1-deficient neutrophils have reduced recruitment to inflammatory sites in vivo (Glogauer et al., 2003; Filippi et al., 2007) and Rac2 is required for neutrophil chemotaxis to the bacterial peptide formyl-met-leu-phe (fMLP) (Carstanjen et al., 2005).

However, primary macrophages that lack Rac1 or Rac2, do not have significant defects in migration (Wells et al., 2004; Wheeler et al., 2006), though other processes are affected such as superoxide production and phagocytosis (Yamauchi et al., 2004). Studies of Cdc42-deficient fibroblastoid cells revealed that polarity and directed cell migration are not exclusively regulated by Cdc42 in these cells (Czuchra et al., 2005). Therefore, though Rac and Cdc42 activities may be important for cell protrusion, during cell migration other GTPases may also have important contributions.

Actin polymerisation dynamics can be affected by Rac and Cdc42 via their effector PAK. It was demonstrated that filamin, an actin filament cross-linking protein localises in membrane ruffles, and is a target of PAK-mediated

phosphorylation (Vadlamudi et al., 2002). In addition, LIMK is a substrate of PAK. The phosphorylation of LIMK led to increased LIMK-catalysed phosphorylation of ADF/Cofilin, which promotes actin filament destabilisation (Edwards et al., 1999).

During migration RhoA regulates the contractile force generation required for tail retraction and to some extent cell body locomotion. The RhoA effector Rho-kinase (ROCK) increases the phosphorylation state of myosin light-chain (MLC) by phosphorylating and inhibiting MLC phosphatase (Kimura et al., 1996). Phosphorylation of MLC increases contractility and this is thought to be the key step in the formation of stress fibres, rather than RhoA promotion of actin polymerisation (Machesky and Hall, 1997). The direct phosphorylation of MLC by ROCK has also been demonstrated (Amano et al., 1996). Leukocytes do not possess stress fibres, but this pathway has been shown to be important for retraction of the tail during migration (Allen et al., 1998; Xu et al., 2003).

Thus, Rac and Cdc42 appear to regulate events at the front of the cell, protrusion and directionality respectively, whereas Rho activity is required in the rear for contraction.

#### 1.2.4.2 Rho GTPases and cell polarisation

The establishment of intracellular PIP<sub>3</sub> gradients, or localised PIP<sub>3</sub> production, contributes to the development of polarity, or gradient sensing (reviewed in (Devreotes and Janetopoulos, 2003)). This has been studied extensively in *Dictyostelium*. The intracellular gradient of this second messenger is created by the reciprocal activities of phosphoinositol 3-kinase (PI3K), which catalyses its production, and PTEN, a PIP<sub>3</sub> phosphatase. In response to a stimulus, PI3K is rapidly recruited to the membrane whereas PTEN dissociates from the membrane. In a gradient of stimulus, or due to local fluctuations in PI3K-PTEN balance, a polarisation of these two activities can be rapidly established, which result in the creation of an intracellular PIP<sub>3</sub> gradient. PI3K activity was, however, not required for the directed migration of




neutrophils to fMLP (Ferguson et al., 2007), indicating that it is not the only means of regulating directionality in migrating cells.

PIP<sub>3</sub> can indirectly activate Rac by activating a number of Rac GEFs, which can be regulated via their PH domain (Rossman et al., 2005). For example Vav1 can be regulated by PIP<sub>3</sub> in vitro (Han et al., 1998), as well as PREX1 (Hill et al., 2005). Furthermore, Rac and Cdc42 could activate PI3K. The p85 subunit of PI3K interacts specifically with GTP-bound Cdc42. This interaction resulted in an increase in the catalytic activity of PI3K in vitro (Zheng et al., 1994). An interaction between GTP-bound Rac1 and the p110 subunit of PI3K has also been demonstrated (Kuroda et al., 1996). Therefore, Rac, Cdc42 and PI3K may be involved in positive feedback regulation of polarised cell protrusion. In addition, PIP<sub>3</sub> can directly promote actin polymerisation with Cdc42 as discussed above (section 1.1).

Rho GTPases also affect cell polarity by their activation of the Par polarity complex, which consists of Par3, Par6 and aPKC (which is the effector protein of the complex) (Mertens et al., 2006). The Par complex regulates numerous aspects of cell polarity through mechanisms that are not yet fully understood (reviewed in (Suzuki and Ohno, 2006)). The Rho GTPase Cdc42 can activate this complex leading to the promotion of cell polarity (Ohno, 2001). Cdc42-mediated aPKC activation leads to the stabilisation of microtubules. APC captures and stabilises microtubules, an activity that is inhibited by phosphorylation by GSK3 $\beta$ . aPKC phosphorylates and inactivates GSK3 $\beta$ , thereby promoting microtubule stabilisation by APC (Etienne-Manneville and Hall, 2001; Etienne-Manneville and Hall, 2003). The Rac GEF Tiam1 has been reported to be an additional component of the Par complex, and thus provides a means by which Rac activity can be polarised in cells, downstream of Cdc42 (Mertens et al., 2006).





**Figure 1.5: Polarisation in migration.** Red: actin filaments are involved in protrusion at the front and retraction in the rear. Green: microtubules are stabilised at the front of the cell, originating from the centrosome (purple), that is also oriented towards the front. Brown: Vesicles from the Golgi apparatus traffic towards the leading edge along microtubules. From (Jaffe and Hall, 2005).

Stabilised microtubules have an increased proportion of detyrosinated tubulin (Glu-MT) (Gundersen and Bulinski, 1988). It was shown that an increase in Glu-MT in response to LPA in 3T3 cells required RhoA (Cook et al., 1998), and was mediated by the RhoA effector mDia (Palazzo et al., 2001). An mDia-EB1-APC complex was also required for stable microtubule formation and normal cell migration (Wen et al., 2004). The Cdc42 and Rac1 effector PAK is also involved in the regulation of microtubule dynamic instability. Stathmin is a microtubule destabilising protein that is inhibited by phosphorylation on Ser16 (Horwitz et al., 1997). PAK (among other kinases) has been shown to catalyse this phosphorylation (Daub et al., 2001).

Finally, Rho GTPases and their effectors may also serve to provide a direct functional link between the actin and microtubule networks. This may be a key process that ensures that the cell protrusive machinery (actin cytoskeleton) is coordinated with the other polarised aspects of the cell. CLIP170 is a microtubule-associated protein that appears to target microtubule plus ends to specific sites (Pierre et al., 1992). IQGAP1 is an effector of Rac and Cdc42 that also binds actin filaments. An association between IQGAP1 and CLIP170 was demonstrated, which provides a physical link between Rac/Cdc42 and microtubules (Fukata et al., 2002).

### **1.3 Integrins in migration**

Cell adhesion is a means by which cells generate traction that can be used to move the cell body forward (Lauffenburger and Horwitz, 1996). Cells form contacts with the extracellular matrix (ECM) via cell surface proteins that interact with ECM components. The integrin family of receptors are transmembrane proteins that specifically recognise and bind components of the ECM and adhesion molecules on other cells via their extracellular domains, and are linked to the cytoskeleton through their cytoplasmic tails. Thus, integrin adhesions can form sites of anchorage for the migrating cell (Hood and Cheresch, 2002).

#### **1.3.1 Integrin structure and signalling**

Integrins are transmembrane glycoproteins that form heterodimers of  $\alpha$  and  $\beta$  chains (Figure 1.6). There are 18  $\alpha$  chain isoforms and 8  $\beta$  chain isoforms. These come together to form 24 distinct dimer pairs, that each have a specific range of ECM ligands (reviewed in (Plow et al., 2000)). Most integrins show a dependence on divalent cations to be able to bind their ligands (Lynn Sigurdson and Lwebuga-Mukasa, 1994). This is conferred by the presence of metal-ion dependent adhesion site (MIDAS) motifs in the extracellular domains of both  $\alpha$  and  $\beta$  chains, which upon binding divalent cations have increased affinity for their ligand (Luo et al., 2007).



**Figure 1.6: General structure of integrin receptors.** Integrins are transmembrane heterodimers composed of  $\alpha$ - and  $\beta$ -subunits. The extracellular domains bind components of the ECM, which results in intracellular signalling via the cytosolic domains. The I-domain and I-like domains are required for ligand binding. The GFFKR sequence in the cytosolic domain of  $\alpha$ -subunits is thought to be involved in 'inside-out' signalling. From Harris et al., 2000.

Integrins are more than just cellular adhesion sites. Ligation of the extracellular domain, or clustering of receptor dimers, induces conformational changes that are transmitted to the intracellular domain (van der Flier and Sonnenberg, 2001). Although the cytosolic domain has no catalytic activity itself, the change in conformation results in the recruitment of a number of cytosolic signalling proteins. The assembly of an integrin signalling complex has been shown to induce changes in the cytoskeleton, cell proliferation and cell survival (reviewed in (Juliano, 2002)).

Ligand binding and receptor clustering of the integrins can be regulated by intracellular events, so-called 'inside-out' signalling (Luo et al., 2007). Structural studies have shown that modulation of integrin affinity for their ligands occurs through changes in the conformation of the cytoplasmic tail of the  $\alpha$ -subunit (Vinogradova et al., 2000). The cytosolic tails of the  $\alpha$ - and  $\beta$ -subunits are in close association. Modulation of the  $\alpha$ -subunit cytosolic tail either by changes in protein interaction and phosphorylation (Blystone et al., 1997) can alter this interaction. The result is a change in conformation of both  $\alpha$  and  $\beta$  chains that affects the extracellular ligand-binding domains. Thus, intracellular signalling events can regulate the 'active' (high affinity) and 'inactive' (low affinity) states of integrins.

This property of integrin affinity to be regulated intracellularly appears to correlate with cell polarity and cell migration. High affinity integrins are known to localise in the actively protruding front of the cell (Kiosses et al., 2001) where new adhesions need to be formed. This regulation is also important for leukocyte recruitment. Leukocytes circulate in blood vessels, only becoming adherent upon certain inflammatory or chemotactic stimuli (Alon and Feigelson, 2002). Chemokine stimulation of leukocytes has been shown to increase their adhesion to integrin ligands (Grabovsky et al., 2000; Campbell et al., 1998). Maintaining integrins in a low-affinity state may be a way to ensure that leukocytes only adhere and transmigrate in regions of inflammation or infection.

### 1.3.2 Integrins and cytoskeletal regulation

During leukocyte recruitment, the rolling leukocyte recognises integrin ligands on the endothelial cells and becomes firmly adherent. Following this, the leukocyte will polarise and adopt a migratory phenotype in order to move through the endothelial cells and towards the site of inflammation. Such a behavioural change requires extensive remodelling of the cytoskeleton. It is now understood that signalling from the integrins plays an essential role in cytoskeletal regulation and the maintenance of a migratory morphology (Moissoglu and Schwartz, 2006). Studies have shown that integrins can

regulate the cytoskeleton by signalling through the Rho GTPases. Lymphocyte spreading in response to activation of the  $\beta_2$  integrin LFA-1 (Leukocyte function-associated Antigen-1) is dependent on the activation of Rac1 (Sanchez-Martin et al., 2004). Rac1 is activated during the  $\alpha_2\beta_1$  integrin-mediated spreading of platelets on collagen (Suzuki-Inoue et al., 2001). The GTPase RhoA is also an important component of integrin-mediated cytoskeletal regulation. However, the role of RhoA appears to be cell type or context dependent. The  $\beta_3$  integrin dependent adhesion of an immature leukaemia cell line required the activation of RhoA (Gao et al., 2005). But in epithelial cells, the localised suppression of RhoA was required for normal adhesion and migration (Tsubouchi et al., 2002).

Cytoskeletal regulation downstream of integrins is mediated by the Rho GTPases. But the GTPase Rap1 has an important role in the activation of integrins. Preventing Rap1 activation inhibits integrin-dependent adhesion of B cells (McLeod et al., 2004) and T cells (Kim et al., 2002). Precisely how Rap1 activates integrins is not known but it may be through interaction with other proteins such as RapL, which binds to both activated Rap1 and the cytoplasmic domain of  $\alpha_L\beta_2$  (Bos, 2005). Interestingly Rap1 also affects cell spreading by localising Rac GEFs such as Vav2 and Tiam1 to the membrane, where they can activate Rac1 (Arthur et al., 2004).

### 1.3.3 Components of the integrin signalling complex

As mentioned above, integrins themselves have no catalytic activity. However, their downstream signalling relies on the recruitment of adapter proteins and protein kinases that are organised into a signalling complex around their cytoplasmic tails (reviewed in (Juliano, 2002)). Prominent components of this signalling complex are the tyrosine kinases such as FAK (Focal-Adhesion Kinase) and the Src family, adapter proteins such as paxillin and Crk and the serine/threonine kinases ERK1/2 and PAK (p21-Activated Kinase) (reviewed in (Giancotti and Ruoslahti, 1999)).

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### 1.3.3.1 Paxillin

The recruitment and activities of the Src family of kinases has been shown to be essential for integrin signalling and associated cellular responses (Suen et al., 1999; Brown et al., 2005). However, the recruitment of these proteins is dependent on the adapter protein paxillin, which can directly associate with integrins (Liu and Ginsberg, 2000). Paxillin is composed of a number of signalling domains that together allow the recruitment and organisation of different signalling molecules.

For example, the LIM domain in the C-terminus of paxillin is known to provide a binding site for the tyrosine phosphatase PTP-PEST (Shen et al., 1998). This is thought to recruit PTP-PEST to adhesion complexes where its substrates such as p130CAS are also localised (Angers-Loustau et al., 1999).

The other key feature of paxillin signalling is its phosphorylation on tyrosine, serine and threonine residues (reviewed in (Turner, 2000)). However it is the tyrosine and serine phosphorylations that are associated with adhesion and migration (Bellis et al., 1997; Iwasaki et al., 2002). Phosphorylation on tyrosine residues could lead to the recruitment of proteins containing SH2 domains, such as Src family kinases. Indeed the phosphorylation of paxillin has been proposed as a crucial step in adhesion complex formation during spreading (Richardson et al., 1997). A protein complex consisting of the adapter protein GIT1, PAK, PIX (PAK interacting exchange factor) and paxillin preferentially localises in regions of cell protrusion and adhesion formation (Manabe et al., 2002). GIT1 is responsible for the localisation of the complex (Manabe et al., 2002), whereas the PAK-PIX module promotes protrusion formation via Rac (Cau and Hall, 2005). The PAK-mediated phosphorylation of paxillin at Ser273 is required for its interaction with GIT1, and the subsequent regulation of normal protrusion and migration in CHO cells (Nayal et al., 2006).

During migration there is a combined requirement for adhesion formation and disassembly within the cell. Paxillin phosphorylation appears to trigger events

necessary for adhesion disassembly. The SH2 domain of the adapter protein Crk has been shown to associate with the tyrosine phosphorylated residues of paxillin, an association that was required for migration of tumour cells (Petit et al., 2000). Crk recruits p130CAS to the complex, which is a substrate of the tyrosine phosphatase PTP-PEST. However, in localising PTP-PEST to the complex, paxillin triggers its own dephosphorylation on tyrosine (Shen et al., 2000) possibly leading to dissociation of bound proteins. In addition, the inactivation of PTP-PEST by tyrosine phosphorylation resulted in the inhibition of migration in MDCK cells (Lu et al., 2006). Thus the phosphorylation of paxillin may be important in maintaining adhesion turnover during cell migration.

#### 1.3.3.2 ERK1/2

The extracellular signal regulated kinases 1 and 2 (ERK1/2) are also important components of integrin signalling. The ERK1/2 signalling cascade is known to effect cell proliferation and differentiation (Roux and Blenis, 2004; Howe et al., 2002), but recent evidence also suggests that ERK signalling is an important factor in migration. ERK is activated when it is phosphorylated on tyrosine and threonine residues by its upstream kinase, MEK. Many studies with MEK inhibitors and expression of inactive MEK mutants have shown that reduction in ERK activity negatively affects cell migration (reviewed in (Huang et al., 2004)). Precisely how ERK1/2 signalling promotes cell migration is still unclear. However studies have shown that it could involve proteins such as Paxillin, Rac, PAK and the protease calpain.

ERK1/2 directly phosphorylates paxillin on Ser83 (Ishibe et al., 2004). This phosphorylation is required for the association of FAK and paxillin induced by hepatocyte growth factor (HGF) in epithelial cells. In a macrophage cell line, ERK1/2-catalysed phosphorylation of serines 126 and 130 of paxillin are also capable of inducing cytoskeletal changes (Cai et al., 2006).

ERK1/2 also directly phosphorylates PAK (Sundberg-Smith et al., 2005), whose downstream effectors include filamin and LIMK (section 1.2). The



interaction between PAK and ERK signalling is complex, possibly involving negative and positive feedback regulation. ERK phosphorylation of paxillin is required for Rac activation (Ishibe et al., 2004), and PAK in turn can activate ERK by phosphorylating MEK1 (Coles and Shaw, 2002). However, ERK also phosphorylates PAK on residues that prevent continued phosphorylation of MEK (Eblen et al., 2002; Eblen et al., 2004; Pullikuth et al., 2005).

ERK may also have an important role in adhesion disassembly during migration. Stimulation of EGF receptor-expressing fibroblasts with EGF induces migration. This migratory response can be blocked by inhibition of ERK or the protease, calpain (Glading et al., 2000). Calpain promotes adhesion disassembly by cleaving proteins of adhesion complexes such as FAK, talin and paxillin (Carragher et al., 1999). ERK appears to be involved in the activation of calpain, since sequestration of its activity from the plasma membrane reduces both calpain activation and de-adhesion (Glading et al., 2001).

#### **1.4 Vav family Rho/Rac GEFs**

The Vav family of Rho/Rac GEFs is a unique family of proteins. Vav GEF activity is regulated by tyrosine phosphorylation, and they possess a number of protein interaction domains that allow them to act as signalling adapters. They provide a link between a number of cell surface receptors and the Rho GTPases. Vav proteins have therefore been proposed to be essential components in the regulation of the cytoskeleton, adhesion and migration.

##### **1.4.1 The Vav family and their structure**

Vav family proteins act as guanine nucleotide exchange factors for members of the Rho family of GTPases. Three members of the family are expressed in mammalian organisms: Vav1, Vav2 and Vav3 (Bustelo, 2000). Vav2 and Vav3 have broad tissue expression patterns but Vav1 is largely restricted to cells of haematopoietic lineage (Bustelo, 2000). Vav1 was the first protein of the family to be discovered. It was the sixth gene identified in a search for

novel oncogenes in the human genome by Katzav and colleagues, and was designated Vav (the sixth letter of the Hebrew alphabet) (Katzav et al., 1989). Subsequently, Vav1 homologues Vav2 (Henske et al., 1995; Schuebel et al., 1996) and Vav3 (Movilla and Bustelo, 1999) were identified. Vav2 has 55% aa sequence homology with Vav1 (Schuebel et al., 1996), whereas Vav3 has 69% aa sequence homology with Vav1, and 66% with Vav2 (Movilla and Bustelo, 1999). All three Vav family members in mammals have an identical domain structure (Figure 1.6).

**Figure 1.7: General domain structure for the Vav family of Rho/Rac GEFs.** Adapted from Zugaza et al., 2002.

The Vav family of proteins are unique in their combination of protein domains (Figure 1.7) and signalling properties. The presence of the DH domain endows them with the catalytic activity required to exchange guanine nucleotide on members of the Rho family. However, they are the only proteins known to date, to combine the DH-PH cassette with the Zinc Finger (ZF) domain (which allows specific protein-protein interactions), and the SH3-SH2-SH3 cassette (Zugaza et al., 2002). The SH3-SH2-SH3 cassette is likely to be responsible for Vav localisation to tyrosine phosphorylated sites, as well as its specific interaction with other signalling proteins, containing proline-rich regions. For example the proline-rich region in the Vav1 C-terminal SH3 domain has been shown to interact with the SH3 domain of the adapter protein Grb2 (Nishida et al., 2001). Such an interaction is thought to be

responsible for the Grb2-dependent localisation of Vav1 in B-cell receptor (BCR) signalling and subsequent Rac activation (Johmura et al, 2003).

#### 1.4.2 Regulation of the Vav proteins

The Vav family proteins transduce signals downstream of a number of cell surface receptors. In addition to the regulation of their catalytic activity by tyrosine phosphorylation, a number of other mechanisms of regulation have also been reported, which reflect the diversity of signalling pathways in which the Vav proteins are involved.

##### 1.4.2.1 Regulation of Vav protein catalytic activity

Vav family catalytic activity is regulated by direct tyrosine phosphorylation. All three members are capable of catalysing guanine nucleotide exchange on Rho family GTPases in a tyrosine-phosphorylation dependent manner (Crespo et al., 1997; Schuebel et al., 1998; Movilla and Bustelo, 1999). The oncogenic form of Vav1 (constitutively active) has a deletion of the N-terminus including the CH-domain (Coppola et al., 1991). Further analyses of Vav1 deletion mutants revealed that the CH and ZF domains were required to maintain inhibition of the catalytic DH domain, which was relieved upon phosphorylation of residues in the acidic region (Zugaza et al., 2002). Three tyrosine residues in the acidic domain have been identified, whose tyrosine phosphorylation state reflects the activity of Vav proteins (Lopez-Lago et al., 2000). Structural studies have shown that an interaction between tyrosine174 and residues in the DH domain could be disrupted by phosphorylation at this site (Aghazadeh et al., 2000). However, mutation of tyrosine174 to a residue that cannot be phosphorylated results in an oncogenic version of Vav, and enhanced phosphorylation of other tyrosine sites (Lopez-lago et al., 2000). Therefore, the regulatory tyrosine174, as well as being responsible for Vav activation, also appears to be involved in the negative regulation of Vav. Interestingly, the oncogenic tyrosine174 mutant of Vav still shows a tyrosine phosphorylation-dependent increase in catalytic activity, suggesting that tyrosine174 is not the only regulatory tyrosine residue (Lopez-Lago et al.,

2000). Tyrosine174 is conserved between Vav family members and so the mechanism of regulation is likely to be similar. In addition, the C-terminal SH3-SH2-SH3 domain though not physically associated with the catalytic domain, may have an important contribution to its regulation, by mediating association with, or localisation to, tyrosine kinases or phosphatases which could regulate it directly (Zugaza et al., 2002).

As discussed (section 1.2), the PH domain of GEFs can also contribute to the catalytic activity and substrate recognition of the DH domain, dependent on the GEF protein. It has been demonstrated that the catalytic activity of Vav proteins can be regulated by phosphoinositide phosphates. Biologically active soluble PI(4,5)P<sub>2</sub> analogues (a PI3K substrate) inhibited the catalytic activity of tyrosine-phosphorylated Vav1, where PI(3,4,5)P<sub>3</sub> (a PI3K product), enhanced the catalytic activity (Han et al., 1998). Furthermore, the binding of PIP<sub>3</sub> to Vav1 occurs via the PH domain.

Negative regulation of Vav proteins can be mediated by dephosphorylation of tyrosine residues. The SH2 domain-containing tyrosine phosphatase-1 (SHP-1) dephosphorylates Vav1 in natural killer cells (Stebbins et al., 2003). Furthermore, in platelets, SHP-1 and Vav were found to be constitutively associated (Jones et al., 2004). In this study, the authors demonstrated that upon stimulation of cells by thrombin, a PKC-mediated phosphorylation of SHP-1 led to its inactivation, coinciding with an increase in tyrosine phosphorylation of Vav1. Another possible candidate is the haemopoietic-specific tyrosine phosphatase PTPN22. Using substrate-trapping PTPN22 mutants, Vav1 was identified as a possible substrate in the Jurkat T-cell line (Wu et al., 2006).

#### 1.4.2.2 Regulation of Vav protein localisation

The PH domain of Vav proteins is thought to localise Vav proteins to sites of PIP<sub>3</sub> production. Macrophages lacking the expression of the phosphoinositide phosphatase SHIP have constitutively membrane-localised Vav1 (Vedham et al., 2005).

The GTPase Rap1 mediates integrin-dependent cell spreading by activating Rac1. It was demonstrated that constitutively active forms of Rap1 selectively associated with Vav2, leading to the recruitment of Vav2 to the plasma membrane during cell spreading (Arthur et al., 2004). The SH2 domain is also required to regulate Vav localisation to specific sites. Vav2 is phosphorylated upon EGF receptor stimulation. But when certain residues in the Vav2 SH2 domain are mutated, Vav2 fails to associate with the EGF receptor and is not phosphorylated in response to EGF stimulation (Tamas et al., 2001). Furthermore, the interaction of Vav with adapters such Grb2 and tyrosine kinases such as ZAP-70 during TCR signalling localise it to glycolipid-enriched membrane fractions (Salojin et al., 2000). In addition, mutation of residues in the PH domain of Vav1 (Palmby et al., 2002) and Vav2 (Booden et al., 2002) reduce but do not abolish their membrane association, indicating the contribution of the cysteine-rich domain and SH3-SH2-SH3 cassette to Vav localisation.

#### 1.4.2.3 Regulation of Vav signalling by ubiquitinylation

Evidence suggests that Vav1 is also targeted for ubiquitinylation and downregulation by the E3 ubiquitin ligase, Cbl. The Cbl family of proteins are important negative regulators of receptor tyrosine kinase signalling, most likely by their action as ubiquitin ligases (Swaminathan and Tsygankov, 2006). Cbl has also been reported as a negative regulator of the non-receptor tyrosine kinase Syk (Ota and Samelson, 1997; Lupher et al., 1998). It has been shown that Cbl can catalyse the ubiquitinylation of Vav1, in a phosphorylation-dependent manner, and that this coincides with a reduction in Vav phosphorylation and an inhibition of its downstream signalling pathways (Miura-Shimura et al., 2003).

#### 1.4.3 Specificity for Rho GTPases

In vitro studies have shown that Vav1 preferentially exchanges guanine nucleotide on Rac1 (Crespo et al., 1997). Vav2 appears to prefer RhoA and

the Rac-like RhoG as substrates (Schuebel et al., 1998). The optimal substrates for Vav3 in vitro are RhoA and RhoG, and to lesser extent, Rac1 (Movilla and Bustelo, 1999). Taken together these results suggest that Vav proteins can discriminate between different Rho GTPases, and in particular prefer Rho and Rac subfamily members to those of Cdc42. It has been shown that, in contrast to Ras-like GTPases, specific binding between Rho/Rac GTPases and Vav is dependent on residues outside the switch regions of the GTPase (Movilla et al., 2001). Furthermore, residues that mediate this interaction are conserved between the Rho and Rac subfamilies but not Cdc42. It remains to be determined if these preferences are maintained in vivo.

#### 1.4.4 GEF-independent functions of Vav proteins

Due to their combination of protein binding domains, it appears that Vav proteins also have a significant role in signalling that is independent of their function as Rho/Rac GEFs. The GEF-independent functions of Vav have been extensively studied in the context of immune receptor signalling in lymphocytes. Upon T-cell receptor (TCR) engagement, signalling from the immune receptor signalling complex (the signalosome) effects cellular responses including, gene regulation via the activation of nuclear factor of activated T-cells (NFAT), cell cycle progression and cytokine production (Turner and Billadeau, 2002). The activation of Vav is known to be a vital component of this pathway, and studies have shown that its involvement is mainly through GEF-independent activities (reviewed in (Katzav, 2004)). It has been demonstrated that GEF activity of Vav is not required for the potentiation of NFAT activity in Jurkat cells (Kuhne et al., 2000). The N-terminal truncation that causes constitutive activation of Vav1 (oncogenic Vav1) failed to potentiate NFAT activity downstream of TCR activation (Wu et al., 1995). This suggested that the CH domain (lost in oncogenic Vav1) has a significant role in this process.

TCR signalling also induces changes in intracellular calcium levels, effected by phosphorylation and activation of PLC- $\gamma$ 1. Activation of PLC- $\gamma$ 1 can be

effected by a pathway dependent on the GEF activity of Vav and requiring PI3K and Rac (Tolias et al., 1995; Bokoch et al., 1996). However, inhibiting PI3K activity only partially reduces PLC- $\gamma$ 1 phosphorylation compared to levels in Vav1<sup>-/-</sup> thymocytes. Furthermore, Vav1 is required for TCR-induced association between the adapter SLP-76 (SH2 domain-containing leukocyte phosphoprotein of 76 kDa) and PLC- $\gamma$ 1 (Reynolds et al., 2002). SLP-76 is a haematopoietic-specific adapter protein that binds to the Vav SH2 domain (Zakaria et al., 2004). Through this association, SLP-76 recruits other proteins required to transduce signals from the TCR, such as the tyrosine kinase ZAP-70 and the Tec family of tyrosine kinases (Leo and Schraven, 2001). Vav association with SLP-76 therefore appears to be essential for the recruitment of other proteins required for TCR signalling. Indeed, in B-cells a specific interaction between Vav1 and ZAP-70 was found to be essential for BCR signalling, using NFAT activation as a read-out (Wu et al., 1997). Furthermore, when this interaction was disrupted, the phosphorylation of Vav1, ZAP-70, SLP-76 and Shc were all reduced.

Interestingly, Vav proteins could affect F-actin reorganisation independent of their GEF activity. Vav1 association with SLP-76 and Nck and subsequent recruitment of VASP was required for integrin-dependent cytoskeletal changes in platelets (Oberfell et al., 2001). A similar pathway is also required in the actin reorganisation in T cells following TCR engagement with antigen presenting cells (APC). Vav1, SLP-76 and the adapter protein Nck were required to colocalise WASp and Cdc42 activity for effective conjugation between T cells and APCs (Zeng et al., 2003). Adapter-mediated recruitment of actin regulating proteins was also demonstrated in macrophages, where a complex including Vav1, SLP-76, Nck, WASp and Ena/VASP localised to sites of actin remodelling during phagocytosis (Coppolino et al., 2001).

#### 1.4.5 Vav proteins in development and disease

Since Vav1 expression is largely restricted to cells of haematopoietic lineage, the study of the biological role of Vav proteins has concentrated on leukocytes. Studies using RNA antisense reported that knocking down Vav1

in haematopoietic stem cells could inhibit the development of erythroid and myeloid type cells in culture (Wulf et al., 1993). However, mice lacking Vav1 expression were shown to develop normal populations of erythroid and myeloid cells (Zhang et al., 1994).

Vav1 does however, have a pronounced effect on the development of lymphocyte populations in mice. Development of T cell populations is mediated through the TCR. Immature T cells (thymocytes) in the thymus progress through developmental stages characterised by the expression of CD4 and CD8. CD4<sup>-</sup>CD8<sup>-</sup> double negative (DN) cells mature into CD4<sup>+</sup>CD8<sup>+</sup> double positive (DP) cells, then into either CD4 or CD8 single positive mature T cell populations. Mice lacking Vav1 have a reduced population of intermediate stage, DP cells, whereas they have normal numbers of DN cells (Turner et al., 1997). This subsequently affects the numbers of the mature single positive populations. The reduction in T cell maturation has been attributed to defective TCR signalling, of which Vav1 is an essential component (Tybulewicz, 2005). Mice lacking Vav1 and Vav2 also have decreased development of mature B cell populations (Doody et al., 2001).

Vav2 and Vav3 are more broadly expressed, and recent studies have highlighted important roles for these Vav isoforms in non-haematopoietic tissues. Angiogenesis is a process requiring endothelial cell migration and morphological regulation leading to the generation of new blood vessels. The process has been attributed to the Eph family of RTK, which signal to Rac1 in order to induce morphological changes in endothelial cells. A recent study demonstrates that Vav2 and Vav3 are required to link Eph receptor ligation to the activation of Rac and subsequent cytoskeletal regulation involved in angiogenesis (Hunter et al., 2006).

Mice lacking Vav3 have cardiovascular dysfunction, displaying both hypertension and tachycardia. The mice were found to have abnormally high levels of renin and Angiotensin-converting enzyme (ACE) in both the plasma and heart (Sauzeau et al., 2006). Renin converts angiotensinogen to angiotensin I, and ACE converts Angiotensin I to angiotensin II. Angiotensin II



promotes vasoconstriction. How Vav3 causes increased levels of renin and ACE is not yet known.

#### 1.4.6 Vav proteins in migration

Rho GTPases are regulators of the cytoskeleton and adhesion, and their coordinated regulation of these two cellular processes effects cell migration (section 1.2). As activators of Rho GTPases the Vav proteins also have important roles in cytoskeletal regulation, adhesion and migration.

Studies have shown that Vav-induced activation of Rho GTPases can induce changes in the actin cytoskeleton. In fibroblasts, N-terminally truncated Vav1 induces the formation of bundled actin filaments (Kranewitter and Gimona, 1999). Vav1 has also been shown to localise in the tips of filopodia of mouse melanoma cells (Kranewitter et al., 2001). Expression of active Vav2 mutants in cells induces membrane ruffling and lamellipodium formation (Abe et al., 2000, Tamas et al., 2003). The expression of constitutively active Vav3 in fibroblasts also induces membrane ruffles and lamellipodia, which are inhibited by co-expression of dominant negative forms of Rho, Rac and Cdc42 (Sachdev et al., 2002). Thus, overexpression studies using active mutants of Vav isoforms have established that Vav proteins can induce reorganisation of the actin cytoskeleton. Interestingly, the Vav proteins appear to regulate filamentous actin structures associated with cell protrusion during migration (membrane ruffles, lamellipodia and filopodia).

Studies of leukocytes have revealed important roles for Vav proteins in migration, particularly the haematopoietic-specific Vav1. Stimulation of lymphocytes with the chemo-attractant SDF-1 resulted in Vav1 tyrosine phosphorylation, and a polarisation of Vav1 localisation to both the leading edge and tail of migrating cells. Furthermore, expression of dominant negative Vav1 caused an inhibition of lymphocyte migration (Vincente-Manzanares et al., 2005). Progenitor haematopoietic cells isolated from Vav1-null mice have a greatly reduced chemotactic response to SDF-1 (Whetton et al., 2003). Vav1 is also activated upon neutrophil stimulation with the

chemokine, fMLP. Neutrophils lacking expression of Vav1 have reduced chemotaxis toward fMLP, and actin polymerisation in response to fMLP stimulation (Kim et al., 2003). In addition, Vav1 has been reported to be required for macrophage chemotaxis to CSF-1 (Vedham et al., 2005).

When ligated by their ECM components, integrins are able to effect changes in the cytoskeleton via activation of the Rho GTPases (section 1.3). Vav proteins also appear to be essential components linking integrins to the Rho GTPases. The ligation of  $\beta_3$  integrin of neutrophils has been shown to activate Vav1 (Zheng et al., 1996). In addition, neutrophils that do not express Vav1 and Vav3 have defects in adhesion and spreading downstream of  $\beta_2$  integrin, coinciding with reduced activation of RhoA, Rac1 and Cdc42 (Gakidis et al., 2004). In lymphocytes, adhesion to the  $\beta_2$  integrin ligand intercellular adhesion molecule-1 (ICAM-1) induces cell spreading and elongation (Rodriguez-Fernandez et al., 1999). Cytoskeletal regulation required to effect these changes was shown to require the activation of Rac1 by Vav1 (Sanchez-Martin et al., 2004). Vav1 has also been shown to interact directly with  $\beta_3$  integrin subunit in the leukaemia cell line K562 upon cell adhesion to fibronectin. And expression of Vav activation mutants in these cells disrupted the activation of RhoA, Rac1 and Cdc42 that occurs upon adhesion (Gao et al., 2005).

Vav proteins therefore provide a link between cell surface receptors and the Rho GTPases, allowing regulated morphological changes that are required for cell migration.

### **1.5 Colony-Stimulating Factor-1 and Macrophages**

Macrophages are essential in the normal development of mammals as well having important roles during inflammation. The macrophage Colony-stimulating factor (CSF-1) stimulates the survival, proliferation and differentiation of macrophage populations. It is also a potent macrophage chemo-attractant and therefore has a significant role in macrophage recruitment to specific tissue sites. Furthermore, CSF-1 may also play a

significant role in regulating the functions of mature macrophages after recruitment.

### 1.5.1 The CSF-1 receptor and intracellular signalling

The CSF-1 molecule is a disulphide-linked homodimer, whose biological activity is lost when the disulphide bond is broken. The molecule signals through the product of the proto-oncogene, c-Fms, a 165 kDa receptor tyrosine kinase. The receptor is a member of the PDGF receptor family. It is composed of five glycosylated immunoglobulin-like extracellular domains and an intracellular tyrosine kinase domain (reviewed in (Yeung and Stanley, 2003)). Upon CSF-1 binding, the receptors form dimers and their intracellular kinase domains become activated. This results in the phosphorylation of numerous tyrosine residues, followed by the formation of an extracellular disulphide bond (Li and Stanley, 1991). Further phosphorylation events result in receptor internalisation, minutes after the initial stimulation.

Phosphorylation of tyrosine residues on the receptor results in the recruitment of numerous proteins such as adapters, non-receptor tyrosine kinases and cytoskeletal proteins resulting in the formation of a multi-protein signalling complex. Studies of the signalling events initiated at the CSF-1 receptor have been carried out by stimulating macrophages with CSF-1 and identifying proteins whose tyrosine phosphorylation state is altered in response, or those proteins that are associated with them (Sengupta et al., 1988; Yeung et al., 1998; Yeung et al., 1992). A variety of proteins have been thus identified, which reflects the varied processes that CSF-1 regulates (reviewed in (Yeung and Stanley, 2003)). Vav1 has also been identified in CSF-1R signalling complexes (Yeung and Stanley, 2003).

### 1.5.2 Biology and role of CSF-1

Purification and isolation of CSF-1 showed it to be a disulphide-linked homodimeric glycoprotein of around 70 kDa (Stanley and Heard, 1977). Two forms of the protein are produced from alternative splicing of the CSF-1

mRNA. The full length mRNA codes for a membrane-associated glycoprotein. The protein is released from the membrane by proteolytic cleavage and is thus secreted in a soluble form. Alternative splicing of the mRNA leads to the loss of the proteolytic cleavage site, resulting in CSF-1 remaining membrane associated and expressed on the cell-surface (reviewed in (Stanley et al., 1997)).

The osteopetrotic mouse (*op/op*) has a mutation in the CSF-1 gene that leads to a severe deficiency in tissue osteoclast and macrophage populations (Wiktor-Jedrzejczak et al., 1990), whereas the circulating monocyte numbers are reported to be normal (Dai et al., 2004). The mice also display toothlessness, defects in male and female fertility and defective neuronal development. These mice have a total absence of the expression of CSF-1 (Wiktor-Jedrzejczak et al., 1990). This demonstrated the widespread and essential role of CSF-1 in the normal development of mice. Reconstitution of normal expression of the CSF-1 gene (both soluble and cell-surface), led to a reversal of all defects of the mouse including a restoration of mononuclear phagocyte populations in tissues (Ryan et al., 2001). When only the cell-surface expression of CSF-1 was restored, macrophage populations in several but not all tissues were restored (Dai et al., 2004). Thus, both the soluble and cell-surface-localised activities of CSF-1 are differentially required in vivo. Coupling of the CSF-1 gene regulatory sequence to the LacZ gene in mice allowed the identification of tissues in which CSF-1 is locally produced. These include ovarian granulosa cells, testicular Leydig cells, and Paneth cells of the small intestine, indicating CSF-1 may also be important for other cell types (Ryan et al., 2001).

How CSF-1 regulates development and tissue remodelling has been studied by analysing its role in regulating changes in the mammary gland of mammals. Breast tissues undergo a series of changes according to the stage of the life cycle, for example puberty or pregnancy (Sapi, 2004). Upon lactation, both CSF-1 and CSF-1 receptor expression are greatly increased in epithelial cells lining the lactating ducts and alveoli (Sapi et al., 1998). Female *op/op* mice have defects in postnatal mammary gland development, with a

majority of females unable to feed their pups (Pollard and Hennighausen, 1994). It was shown that these mice also have a lack of macrophage populations in breast tissues, which when restored lead to normal breast development (Gouon-Evans et al., 2000). The authors suggested that CSF-1-mediated macrophage and eosinophil recruitment was a key factor in post-natal breast development. CSF-1-dependent changes in tissue remodelling during developmental changes are therefore affected by the differentiation and recruitment of macrophage populations.

### 1.5.3 CSF-1 and macrophage functions

CSF-1 regulates the function of the mature mononuclear phagocytes and promotes macrophage functions required to mediate the inflammatory response. CSF-1 is required for monocyte production of TNF $\alpha$  (Warren and Ralph, 1986) and CSF-1-treatment of monocytes causes an upregulation of the pro-inflammatory cytokines IL-12 and IL-18, as well as the cell surface proteins CD14, CD23 and CD64 (Ji et al., 2004). CD14 can act as a receptor for LPS. CD23 and CD64 are Fc $\epsilon$ RII and Fc $\gamma$ RI receptors that mediate IgE and IgG- dependent phagocytosis respectively. CSF-1 also stimulates macropinocytosis in macrophages, which is an important process during antigen presentation (Murray et al., 2000).

### 1.5.4 CSF-1 and macrophage migration

CSF-1 induces recruitment of macrophages to sites of inflammation (Le Meur et al., 2002). The ability of CSF-1 gradients to stimulate directed migration of monocytes was demonstrated by Wang et al. (Wang et al., 1988). This was confirmed by direct observation of macrophages migrating in a gradient of CSF-1 (Webb et al., 1996). Insights into how CSF-1 could regulate macrophage migration were provided by in vitro studies which showed that CSF-1 could regulate morphological changes in the immortalised mouse macrophage cell line Bac1.2F5 (Boocock et al., 1989). CSF-1-deprivation for 16-24 hours caused cells to withdraw adhesions and become rounded. Upon re-addition of CSF-1 membrane ruffles and cell spreading were both induced

within 5 minutes. This demonstrates that CSF-1 can regulate the actin cytoskeleton in macrophages, and that could thereby induce macrophage migration. CSF-1-induced membrane ruffling has been shown to require Rac1 (Allen et al., 1997) and Cdc42 (Cox et al., 1997). Furthermore, the activation of Rac upon CSF-1 stimulation has been reported in macrophages (Vedham et al., 2005) and osteoclasts (Sakai et al., 2006). CSF-1-induced morphological changes are therefore regulated by the Rho GTPases.

It has been postulated that PI3K-catalysed production of the second messenger  $PIP_3$  is a key factor in the establishment of cell polarity, and thus, cell migration (reviewed in Devreotes and Janetopoulos, 2003). Indeed, PI3K $\lambda$  was shown to be required for macrophage chemotaxis to MCP-1 (Jones et al., 2003). CSF-1-induced ERK1/2 activation is blocked by the PI3K inhibitors LY294002 and wortmannin (Bhatt et al., 2002). Therefore, chemoattractant-stimulated PI3K activation may be important for the induction of migration. CSF-1 signalling could therefore stimulate macrophage migration by the induction of polarity and cell protrusion via activation of Rho GTPases and PI3K.

The regulation of adhesion is also essential to produce net cell translocation. And there is evidence to suggest that CSF-1 can influence adhesion structures in macrophages. Adherent macrophages often contain adhesion structures called podosomes. These have a central core of bundled actin filaments, surrounded by integrins and integrin-associated proteins such as paxillin and FAK (reviewed in Linder and Kopp, 2005; Evans and Matsudaira, 2006). The biological role of these structures appears to be important in invasion and matrix degradation, which may have more relevance for migration in vivo. In migrating macrophages in vitro, podosomes decorate the leading edge of the cell (Evans et al., 2003). This implies a role for podosomes in migration in culture. CSF-1 stimulation was shown to cause an increase in podosomes in BMM, which was dependent on PI3K activity (Wheeler et al., 2006). In addition, CSF-1 has been shown to cause FAK relocalisation to focal adhesions, and was required for CSF-1-mediated cell spreading (Rovida et al., 2005).

CSF-1 can therefore recruit macrophages to particular sites in vivo by acting as a macrophage chemoattractant. And it can stimulate macrophage migration in culture by inducing morphological changes regulated by Rho GTPases.

## **1.6 Aims**

The aim of this investigation was to study the role of Vav proteins in migration and morphological regulation, using primary macrophages as a model system.

The Rho family of GTPases are integral to the regulation of the cytoskeleton. As Rho/Rac family GEFs, Vav proteins have been identified as essential components linking a variety of cell surface receptors to the Rho GTPases. It is therefore possible that Vav proteins are required as Rho GEFs during macrophage morphological changes and migration.

The ability to regulate cell morphology and migrate in response to extracellular stimuli is an essential property of macrophages. The cells circulate in blood vessels and in response to specific extracellular cues, adhere to the endothelium, and then migrate towards sites of inflammation or injury. Macrophages have therefore been used to study migration and morphology, and the role of Vav proteins therein.

Many of the previous investigations of Vav function have utilised overexpression approaches or cell lines. These approaches may produce results which are an artefact or may not reflect the function of these proteins in a physiologically relevant context. In this investigation, macrophages have been derived from mice lacking expression of single or all three isoforms of Vav (kindly provided by Dr Martin Turner, Babraham Institute, Cambridge).

The role of Vav proteins in regulating macrophage migration and their morphological and signalling responses to adhesion and the chemo-attractant CSF-1 have been investigated by comparing Wt and Vav-deficient macrophages.



## **2. Methods**

### **2.1: Cell Culture**

#### **2.1.1: Materials**

Materials	Source	address
RPMI 1640 + 1% L-glutamine	Invitrogen	<a href="http://www.invitrogen.com">www.invitrogen.com</a>
Dulbecco's Modified Eagle Medium	Invitrogen	<a href="http://www.invitrogen.com">www.invitrogen.com</a>
Non-essential amino acids (100x)	Invitrogen	<a href="http://www.invitrogen.com">www.invitrogen.com</a>
Sodium pyruvate (100x)	Invitrogen	<a href="http://www.invitrogen.com">www.invitrogen.com</a>
Fetal Calf Serum (FCS)	Helena Bioscience	<a href="http://www.helena-biosciences.com">www.helena-biosciences.com</a>
Murine Colony Stimulating Factor (m-CSF)	R&D Systems	<a href="http://www.RnDSystems.com">www.RnDSystems.com</a>
Penicillin/Streptomycin (100x)	Invitrogen	<a href="http://www.invitrogen.com">www.invitrogen.com</a>
Trypsin-EDTA	Invitrogen	<a href="http://www.invitrogen.com">www.invitrogen.com</a>
Versene-EDTA	Invitrogen	<a href="http://www.invitrogen.com">www.invitrogen.com</a>

Table 2.1: List of materials and reagents

#### **2.1.2: Culture Media**

Macrophage Growth Medium    RPMI 1640 + 1% L-glutamine  
    10% FCS  
    Penicillin/Streptomycin (1x)  
    Non-essential amino acids (1x)  
    Sodium pyruvate (1x)  
    0.24 mM  $\beta$ -mercaptoethanol  
    supplemented with 10% LCCM  
    (LCCM: see section 2.1.9)

Macrophage Starve Medium    As above, without LCCM

Medium for L-cells

Dulbecco's Modified Eagle Medium  
Penicillin/Streptomycin (1x)  
10% FCS

### 2.1.3: Vav-deficient mice

Single isoform Vav-null mice (Vav1<sup>-/-</sup>: Turner et al., 1997; Vav2<sup>-/-</sup>: Doody et al., 2001; Vav3<sup>-/-</sup>: Fujikawa et al., 2003) were generated by targeted disruption of the respective Vav gene in an exon containing part of the Dbl homology domain. Targeted gene disruption was achieved by homologous recombination with vectors containing positive selection markers flanked by genomic fragments of the vav gene, transfected into embryonic stem cells by electroporation. Recombinant stem cells were purified by positive selection. In each case, the absence of full-length and truncated forms of Vav protein was confirmed by Western blotting. Vav1/2/3<sup>-/-</sup> mice were generated by cross-breeding of the single deficient mice, and then maintained as a strain. All Vav-deficient mice populations were viable.

### 2.1.4: Bone marrow cells isolation and differentiation

Mice legs were provided by Dr Elena Vigorito and Dr Helen Reynolds from the laboratory of Dr Martin Turner, Babraham Institute, Cambridge, UK. Mouse legs were placed in cold PBS in a plastic Petri dish. Skin was removed by securing the foot with forceps and pulling the skin away. The muscle and cartilage tissues were removed thoroughly and the clean bones were placed into another Petri dish of PBS. 10 mL of macrophage starve medium was added to a 50 mL falcon tube (for easy access with a syringe). Using a 25G syringe needle both ends of the bones were pierced, then 5 mL of the medium from the falcon tube was used to repeatedly flush the marrow out of the bone via the holes at each end into the falcon tube. This was done until the bone appeared white. The bone marrow cell suspension was passed through the syringe repeatedly to break up cell clumps, resulting in a turbid suspension of bone marrow cells.

An aliquot of this suspension was taken and total cells counted using a haemocytometer. The suspension was centrifuged at 1000 rpm (ALC PK130R centrifuge) for 5 minutes, and re-suspended to  $1 \times 10^6$  cells/mL, in macrophage growth medium. Cells were plated on 10 cm diameter bacteriological dishes (1029 BD Falcon) at 37°C, 5% CO<sub>2</sub> for 3 days.

#### 2.1.5: Preparation of cells for liquid nitrogen storage

Cells in suspension were counted, centrifuged at 1000 rpm (ALC PK130R) for 5 min and resuspended at  $5 \times 10^6$  cells/mL, in ice-cold macrophage starve medium with added DMSO to a final concentration of 10%. The cell suspension was immediately aliquoted into 1.8 ml cryovials (NUNC) on ice. Tubes were placed in a polythene container at -80° C overnight, then into liquid nitrogen for long-term storage.

#### 2.1.6: Culture of BMM

For all further experiments, cells were recovered from liquid nitrogen storage by thawing and adding to 10 mL of warm macrophage starve medium. The suspension was centrifuged at 1000 rpm (ALC PK130R) for 5 minutes and the supernatant discarded. Cells were resuspended in 5 mL macrophage growth medium. Cells were plated on 5 cm bacteriological dishes (1007 BD falcon) for 4 days. This yielded a near-confluent dish of adherent macrophages on the 7<sup>th</sup> day after the initial bone marrow isolation.

#### 2.1.7: Preparation of cells for experimentation

Day 7 macrophages were detached from the bacteriological culture dishes using versene-EDTA warmed to 37°C. This is a chelating agent, which sequesters divalent cations such as Mg<sup>2+</sup> and Mn<sup>2+</sup> from solution. These cations are required by certain integrins in their active conformation (van der Flier and Sonnenberg, 2001). The medium was removed and 5 mL warmed versene-EDTA was added. The dish was then placed in the incubator and kept at 37°C for 5 to 10 minutes. The remaining loosely adhered

macrophages were dislodged with pipetting. The cell suspension in versene-EDTA was added to 5 mL macrophage starve medium and centrifuged at 1000 rpm (ALC PK130R) for 5 minutes. The supernatant was discarded and the cells resuspended in 10 mL warmed macrophage growth medium. An aliquot was taken to determine cell density using a haemocytometer. Cells were then diluted and/or seeded at the desired concentration according to the requirements of the experiment.

#### 2.1.8: Stimulation with CSF-1

Before stimulation, cells were cultured for at least 24 hours in macrophage growth medium. For biochemical studies BMM were seeded at a density of 30,000 cells/cm<sup>2</sup>. For immunofluorescence staining experiments, cells were seeded at a minimum density of 22,500 cells/cm<sup>2</sup>.

Cells were starved of CSF-1 by first washing once in warm macrophage starve medium, before culturing for 16 hours at 37°C and 10% CO<sub>2</sub> in macrophage starve medium. The medium from each well was then aspirated and added to a Falcon tube containing CSF-1 (appropriate for a 1/300 dilution, 33 ng/mL). This was mixed by pipetting and added back to the cells for the indicated time.

#### 2.1.9: Culture of L929 fibroblasts and collection of conditioned medium

Macrophages are dependent on CSF-1 for their survival and proliferation (Tushinski et al., 1982). Medium conditioned by L929 murine fibroblasts contains CSF-1 (Das and Stanley, 1982), and can therefore be used as a source of CSF-1 for the differentiation and culture of macrophages.

Cells were cultured in T75 tissue culture flasks, passaging twice weekly. Cells were washed in warmed PBS, then detached using trypsin-EDTA (2 mL per flask) and incubated at 37°C. After 5 minutes, the cell suspension was made up to 10 mL with additional medium, and homogenised by pipetting. This cell

suspension was diluted 1 in 10 to a final volume of 20 ml and re-plated into fresh T75 flasks.

For conditioning of the medium, the L929 cells were allowed 24 hours to adhere to the flask. The ventilation via lids of flasks was then stopped. These cells were left to condition the medium for 3 weeks at 37°C. After 3 weeks, the medium was decanted into 50 mL falcon tubes. The tubes were centrifuged at 1000 rpm (ALC PK130R) for 5 minutes to pellet any cells or debris. The supernatant was then filtered (pore size 0.2 µm, Millipore Stericup). The filtered medium (L929 cell-conditioned medium, LCCM) was aliquoted into Falcon tubes and stored at 4°C for a maximum of 4 months.

## 2.2: Protein Biochemistry

### 2.2.1: Materials

Materials	Source	address
Biorad protein assay reagent	Bio-Rad Laboratories Ltd	<a href="http://www.biorad.com">www.biorad.com</a>
ECL reagents	Amersham Pharmacia	<a href="http://www.ameshambiosciences.com">www.ameshambiosciences.com</a>
Glutathione-sepharose beads	Amersham Biosciences	<a href="http://www.ameshambiosciences.com">www.ameshambiosciences.com</a>
Protein A and protein G sepharose beads	Amersham Biosciences	<a href="http://www.ameshambiosciences.com">www.ameshambiosciences.com</a>
XCell Mini Cell PAGE kit	Bio-Rad Laboratories Ltd	<a href="http://www.biorad.com">www.biorad.com</a>
NuPAGE 4-12% Bis-Tris gradient gel	Invitrogen	<a href="http://www.invitrogen.com">www.invitrogen.com</a>
NuPAGE MOPS-SDS Running Buffer (20x)	Invitrogen	<a href="http://www.invitrogen.com">www.invitrogen.com</a>
NuPAGE MES-SDS Running Buffer (20x)	Invitrogen	<a href="http://www.invitrogen.com">www.invitrogen.com</a>
Biorad Precision Plus Protein Standards (stained)	Bio-Rad Laboratories Ltd	<a href="http://www.biorad.com">www.biorad.com</a>

Table 2.2: Materials used for biochemical studies

2.2.2: Antibodies

Primary antibodies				
Antibody	species	Use	Source	Address
$\beta$ -actin	mouse	1/4000	Sigma-aldrich	www.sigma-aldrich.com
Fms (CSF1R)	rabbit	1/1000	Upstate Biotechnology	www.upstatebiotech.com
p-ERK1/2	rabbit	1/1000	Cell Signaling Technology	www.cellsignal.com
Paxillin	mouse	1/1000	BD Transduction Laboratories	www.bdbiosciences.com
p-Paxillin (pY118)	rabbit	1/1000	Biosource	www.biosource.com
Rac1	mouse	1/1000	Upstate Biotechnologies	www.upstatebiotech.com
RhoA	mouse	1/500	Santa-Cruz Biotechnology	www.scbt.com
$\beta$ -tubulin	mouse	1/2000	Sigma-aldrich	www.sigma-aldrich.com
phospho-tyrosine (4G10)	mouse	1/2000	Upstate Biotechnologies	www.upstatebiotech.com
Vav1	rabbit	WB:1/1000 IP:2 $\mu$ g/mL	Santa-Cruz Biotechnology	www.scbt.com
Vav2	rat	WB:1/1000 IP:2 $\mu$ g/mL	Babraham Bioscience Technologies	www.babraham.com
Vav3	rabbit	WB:1/1000 IP:2 $\mu$ g/mL	Upstate Biotechnologies	www.upstatebiotech.com
Horseradish peroxidase-conjugated Secondary Antibodies				
Anti-mouse		1/2000	Amersham Biosciences	www.amersham.com
Anti-rabbit		1/2000	Amersham Biosciences	www.amersham.com
Anti-rat		1/2000	Amersham Biosciences	www.amersham.com

Table 2.3: List of antibodies used for biochemical study

2.2.3: Buffers and solutions

NP-40 Lysis buffer	10 mM TRIS pH7.5 5 mM EDTA 150 mM NaCl 0.5% NP-40 0.5% sodium pyrophosphate (w/v)
RHEB (Rho-Extracting lysis Buffer)	50 mM TRIS pH7.5 2 mM EDTA 500 mM NaCl 10 mM MgCl <sub>2</sub> 0.1% SDS (w/v) 1% Triton X-100 (v/v) 10% glycerol (v/v) 0.5% sodium deoxycholate (w/v)
Phosphatase and protease inhibitor supplements	1 mM NaF 1.6 µg/mL Aprotinin 1 mM PMSF 1 mM DTT 0.1 mM Na <sub>3</sub> VO <sub>4</sub> 1 µg/mL Leupeptin
4 x protein sample buffer	0.25 M TRIS pH7.5 8% SDS (w/v) 40% glycerol (v/v) 2.82 M β-mercaptoethanol 0.2% bromophenol blue (w/v)
10 x Running buffer	0.248 M TRIS pH7.5 1.92 M glycine 1% SDS (w/v)
10 x Transfer buffer	0.248 M TRIS pH7.5 1.92 M glycine
NuPAGE MOPS SDS Running buffer (20x)	1.0 M MOPS 1 M TRIS 69.3 mM SDS 20.5 mM EDTA
NuPAGE MES SDS Running buffer (20x)	1.0 M MES 1.0 M TRIS 69.3 mM SDS 20.5 mM EDTA
TBS-Tween	50 mM NaCl 25 mM TRIS pH7.5 0.1% Tween-20 (v/v)

Milk Blocking buffer	50 mM NaCl 25 mM TRIS pH7.5 0.1% Tween-20 (v/v) 5% Non-fat milk powder (w/v) (2% Non-fat milk powder for antibody dilutions)
BSA Blocking buffer	50 mM NaCl 25 mM TRIS pH7.5 0.1% Tween-20 (v/v) 5% BSA (w/v)
Membrane stripping solution	78.1 mM TRIS pH7.5 1% SDS (w/v) 0.675% $\beta$ -mercaptoethanol (v/v)
STE buffer	10 mM TRIS pH7.5 150 mM NaCl 1 mM EDTA
Coomassie Staining solution	2.5 mg/mL Coomassie brilliant Blue 40% methanol (v/v) 10% acetic acid (v/v)
Coomassie Destain solution	40% ethanol (v/v) 10 % acetic acid (v/v)
SDS PAGE solutions	See Tables 2.4 and 2.5

#### 2.2.4: Cell Lysis

Dishes/wells were washed with cold PBS and immediately placed on ice. Cold lysis buffer was added to each dish/well and left for 5 minutes. Cold cell scrapers were used to collect the lysate. The lysates were collected into cold microfuge tubes and centrifuged for 8 minutes at 14,000 rpm (Eppendorf 5417R). The supernatant was transferred to fresh cold microfuge tubes.

#### 2.2.5: Protein Assay

Protein assay reagent (BioRad) was diluted 1/5 in water. A standard curve of protein concentrations was made by preparing 2, 4, 8, 16 and 20  $\mu\text{g/mL}$  BSA solutions in 1000  $\mu\text{L}$  assay reagent in polystyrene cuvettes. For determination of lysate protein concentration, 5  $\mu\text{L}$  of lysate was added to 995  $\mu\text{L}$  of assay reagent. Cuvettes were briefly vortexed and the absorbance of samples and



standards at 595 nm was recorded. Sample protein concentrations were determined by reference to the standard curve.

### 2.2.6: SDS Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gels were prepared as shown in Table 2.5 using solutions shown in Table 2.4. using the Bio-Rad Mini-Protean Cell system. Alternatively, where indicated the NuPAGE Bis-Tris 4-12% gradient gel system (with X-cell Sure-Lock tanks, Invitrogen) was used to resolve proteins. Protein samples were loaded into the wells in parallel with molecular weight standards. Proteins were resolved by applying a voltage of 90 to 120 V for approximately 1.5 hours.

Solution 1	0.75 M TRIS pH 8.8, 0.29% SDS
Solution 2	37:5:1 acrylamide:bisacrylamide (40 % w/v solution)
Solution 3	0.25 M TRIS pH 6.8, 0.2% SDS

Table 2.4: Solutions for preparing polyacrylamide gels

Solution	8%	10%	12%	15%	Stacking gel
1 (mL)	3.75	3.75	3.75	3.75	_____
2 (mL)	1.6	2	2.4	3	0.22
3 (mL)	_____	_____	_____	_____	1.22
water (mL)	2.15	1.75	1.35	0.75	0.95
13 % APS (μL)	37.5	37.5	37.5	37.5	22.5
TEMED (μL)	10.5	10.5	10.5	10.5	5.6

Table 2.5: Preparation of polyacrylamide gels

### 2.2.7: Western blotting

Once proteins had been sufficiently resolved, the gel was removed and submerged in transfer buffer for 1 to 2 minutes. PVDF membrane (Immobilon-P, Millipore) was hydrated by soaking in methanol and washed with de-ionised water. The membrane was then briefly soaked in transfer buffer before being placed on top of the gel. The gel and membrane were then placed within filter paper, and sponges soaked in transfer buffer, and closed within the transfer cassette. This was placed in the transfer tank, which was filled with transfer buffer. Transfer of proteins was initiated by applying a voltage of 60 to 70 V for 1.5 hours (or at 4°C overnight at 30 V). The membrane was removed from the cassette and incubated with agitation in blocking buffer for 1 hour at room temperature. The membrane was then incubated with agitation in primary antibody solution made up in blocking buffer overnight at 4°C. Following this incubation, the membrane was washed in TBS-Tween for at least 30 minutes, changing the solution 3 to 4 times. The membrane was incubated with HRP-conjugated secondary antibody for 1 hour at room temperature, followed by washing in TBS-Tween for up to 1 hour. The membrane was placed in ECL reagents for up to 2 minutes, before being drip-dried and covered in Saran wrap. X-ray films were exposed to the membranes for a range of times and then developed.

### 2.2.8: Membrane stripping

Membranes were placed in strip buffer with agitation at 65°C for 20 minutes. The strip buffer was removed and after a few short washes in TBS-Tween, was replaced in TBS-Tween and incubated for a further 5 minutes at 65°C. Following this, the membrane was washed for 30 minutes in TBS-Tween, changing the solution at least three times. Membranes were incubated in blocking solution for 30 minutes at room temperature, before being incubated with primary antibody solution.

### 2.2.9: Densitometric analysis of Western blots

Developed X-ray films were scanned using a BioRad GS-800 densitometer, which recorded 1-dimensional band intensities. Bands of interest were then analysed using the accompanying software using a global background correction. Densitometric values were then exported to Microsoft Excel where they were analysed further.

### 2.2.10: Immunoprecipitation

According to the antibody being used, protein A or protein G sepharose beads were washed in cold NP-40 lysis buffer (no supplements) 3 times. 25  $\mu$ L of washed bead slurry was dispensed into 1.5 mL microfuge tubes and kept on ice. Finally, 2  $\mu$ g of the antibody was added to the beads slurry.

Cells were lysed in NP-40 lysis buffer supplemented with protease and phosphatase inhibitors but not DTT. Lysis was carried out as described (section 2.2.4). After centrifugation, 15  $\mu$ L of the supernatant was added to 5  $\mu$ L of 4x protein sample buffer and kept on ice. The rest of the supernatant was aspirated (carefully avoiding the pellet) and added to the tubes containing sepharose beads and antibody. The tubes were inverted several times to ensure mixing of the contents. The tubes were placed on a rotator at 4°C for 3 hours. Tubes were then centrifuged for 30 seconds at 4°C, to a maximum rpm of 14000 (Eppendorf 5417R). The supernatant (lysate) was aspirated and discarded. The beads were washed 3 times in 1 mL volumes of cold NP-40 lysis buffer (no supplements). Following the final wash, the supernatant was discarded and 15  $\mu$ L of 4x protein sample buffer was added. These samples along with those prepared before the incubation with antibody (Whole Cell Lysates (WCL)) were heated to 100°C for 10 minutes, before storing at -20°C.

### 2.2.11: RhoA and Rac1 GST Pull-Down assay

#### (a) Purification of the GST fusion proteins

PGEX plasmids coding for GST-Rhotekin-RBD and GST-PAK1-PBD were provided by Dr Martin Schwartz and Dr John Collard respectively. *E.coli* strain BL21 transformed with the above constructs were stored as glycerol stocks (at -80°C). They were scraped with a sterile pipette tip and used to inoculate 100 mL of LB medium (with 100 µg/mL Ampicillin). The culture was incubated overnight at 37°C with shaking. The overnight culture was then diluted 1/20 into 500 mL of LB media (with 100 µg/mL Ampicillin). This culture was incubated at 37°C for at least one hour with shaking, until the optical density at 600 nm was between 0.7 and 1.

IPTG was added to a final concentration of 0.45 mM to activate expression of GST constructs in the bacterial cells via the Lac UV5 promoter. The culture was incubated at 30°C for a further 2 hours. The culture was then divided into 50 mL Falcon tubes on ice. The tubes were centrifuged at 3000 rpm (Beckman centrifuge J-6M/E) at 4°C for 20 minutes. The supernatant was decanted off and discarded, and the pellets placed in -80°C storage for at least one night.

To isolate the GST-fusion proteins, pellets were re-suspended in cold STE buffer, supplemented with 1 mM PMSF. Multiple pellets were sequentially re-suspended in the same volume of STE buffer (being equal to 0.75 mL buffer per pellet). Once re-suspended, lysozyme solution was added to a final concentration of 100 µg/mL. The mixture was inverted 6 to 8 times with occasional mixing and placed on ice for 15 minutes (30 minutes during the isolation of the GST-Rhotekin-RBD). DTT was added to a final concentration of 5 mM and mixed by inverting. Tween-20 and SDS were added to final concentrations of 1% and 0.03% respectively, and then mixed by inversion. The lysate was divided into 1 mL microfuge tubes on ice and centrifuged at 14000 rpm (Eppendorf 5417R) at 4°C for 30 minutes. To prepare the pull-down assay beads (PD beads) the supernatant was collected, pooled and

incubated with glutathione-sepharose beads (pre-washed in STE buffer), for 10 to 15 minutes at room temperature with gentle agitation.

The PD beads were washed in STE buffer three times, stored at 4°C and used the following day. 5 µL of the beads were boiled with 5 µL protein sample buffer. This sample was loaded together with BSA protein concentration standards onto a 12% polyacrylamide gel and resolved by electrophoresis. Amounts of purified GST-fusion proteins were estimated by Coomassie blue staining against BSA protein concentration standards (section 2.2.12).

#### (b) Pull-down assay

The PD beads were aliquoted into volumes according to the estimated amounts of bound GST-fusion protein (from Coomassie blue staining). Cells for the PD were lysed as described (section 2.2.4) but with some modifications: RHEB lysis buffer (with added 0.5% β-mercaptoethanol) was used to solubilise the cells, and the lysates were centrifuged for 10 minutes at 14,000 rpm (Eppendorf 5417R) and 4°C.

Following centrifugation 15 µL of lysate (supernatant) was taken and added to 5 µL of 4x protein sample buffer (WCL) and heated to 100°C for 5 min and stored at -20°C. The rest of the lysate was carefully aspirated avoiding the pellet and added to the beads. Tubes were inverted to ensure mixing of contents and then placed on a rotator at 4°C for 1.5 hours.

Tubes were centrifuged briefly (4°C, 30 seconds, 14000 rpm (Eppendorf 5417R)). The beads were then washed with 1 mL volumes of cold lysis buffer (RHEB lysis buffer, no supplements). After washing, the supernatant of the final wash was aspirated carefully, leaving approximately 30 µL above the beads. 10 µL of 4x protein sample buffer was added to all tubes. Samples were then heated to 90 to 100°C for 10 minutes.

Pull down samples, and WCL, were resolved by 12% SDS-PAGE. The level of activation of the respective GTPases was analysed by western blotting both PD samples and WCL samples using antibodies specific for the respective GTPase. Changes in the level of GTPase precipitated (Active) were compared to the levels in WCL (Total) samples by densitometry (section 2.9.9).

#### 2.2.12: Coomassie blue staining

Polyacrylamide gels were incubated, with shaking, in Coomassie stain solution for 30 minutes at room temperature. Gels were then washed several times in destain solution, before a final overnight wash. Finally, gels were washed in distilled water and dried.

#### 2.2.13: Spreading Analysis

BMM were seeded onto 10 cm tissue culture dishes (Greiner) for the indicated times in a volume of 5 ml per dish. Cells were incubated for the indicated times in 37°C and 10% CO<sub>2</sub>. The cells were lysed as described (section 2.2.4), using the RHEB lysis buffer for Rho GTPase pull-down assays; with the NP-40 lysis buffer for other biochemical analyses.

### **2.3: Cell Biology**

#### 2.3.1: Reagents and Buffers

DAKO fluorescent  
mounting medium

[www.dakocytomation.us](http://www.dakocytomation.us)

Fixing buffer

3.7% formaldehyde in  
PBS

Blocking buffer

20% goat serum  
in PBS

Imaging wax

1:1:1 mixture of Lanolin,  
Vaseline and paraffin  
wax2.3.2: Immunofluorescence staining and flow cytometry reagents

Primary Antibodies				
antibody	species	Use/Dilution	Source	address
F4/80 antigen	rat	1/100	ebioscience	<a href="http://www.ebioscience.com">www.ebioscience.com</a>
RPE-CD29( $\beta$ 1 integrin)	hamster	1/20	serotec	<a href="http://www.ab-direct.com">www.ab-direct.com</a>
RPE-CD18( $\beta$ 2 integrin)	rat	1/40	BD Pharmingen	<a href="http://www.bdbiosciences.com">www.bdbiosciences.com</a>
FITC-CD61( $\beta$ 3 integrin)	rat	1/100	Biolegend	<a href="http://www.biolegend.com">www.biolegend.com</a>
Non-antibody fluorophore-conjugated reagent				
TRITC-phalloidin		0.2 $\mu$ g/mL	Sigma-aldrich	<a href="http://www.sigma-aldrich.com">www.sigma-aldrich.com</a>

Table 2.6: Antibodies used for immunofluorescence staining and flow cytometry

2.3.3: Image Acquisition equipment

Equipment	Manufacturer	Address
Axiophot 135 Microscope	Zeiss	<a href="http://www.zeiss.com">www.zeiss.com</a>
LSM510 Confocal microscope	Zeiss	<a href="http://www.zeiss.com">www.zeiss.com</a>
Eclipse TE 2000-E microscope	Nikon	<a href="http://www.nikon-instruments.com">www.nikon-instruments.com</a>
KPM1E/K-S10 CCD camera	Hitachi Denshi	<a href="http://www.hda1.com">www.hda1.com</a>
ORCA-ER CCD camera	Hamamatsu Photonics	<a href="http://www.hamamatsu.com">www.hamamatsu.com</a>

Table 2.7: Image acquisition equipment

2.3.4: Image acquisition and analysis software

Software	Source	Address
AQM Advance 6	Kinetic Imaging Ltd	<a href="http://www.kineticimaging.com">www.kineticimaging.com</a>
LSM510	Zeiss	<a href="http://www.zeiss.com">www.zeiss.com</a>
MetaMorph 5.01	Universal Imaging Systems	<a href="http://www.universal-imaging.com">www.universal-imaging.com</a>
Motion Analysis	Andor Technology	<a href="http://www.andor.com">www.andor.com</a>
Mathematica 5.0	Wolfram Research	<a href="http://www.wolfram.com">www.wolfram.com</a>

Table 2.8: Image acquisition and analysis software

2.3.5: Random migration

Cells were detached and  $4 \times 10^4$  cells were seeded in 35mm tissue culture dishes (NUNC) in macrophage growth medium. Cells were incubated for 24 hours in 10% CO<sub>2</sub> and 37°C. Dishes were placed on a pre-heated stage (set at 37°C) within a chamber that was humidified by means of a water-well and maintained at 10% CO<sub>2</sub> for the duration of the recording. For experiments with bacterial plastic dishes, cells were seeded in 50mm dishes (1007, BD Falcon).

An appropriate field of cells was chosen that had cells of a uniform density. Clusters of cells were avoided, to limit the influence of cell-cell interactions and obstructions on single cell migration. According to the requirements of the experiment, X10 and X20 objective lenses were utilised. Total number of frames, and frame rate were also set according to the experiment. Cells were filmed using a Zeiss Axiovert 135 microscope and KPM 1E/KS10 CCD Camera.



### 2.3.6: Dunn Chamber Chemotaxis assay

$6 \times 10^4$  BMM were seeded on a 22mm x 22mm glass coverslip contained within a 35 mm tissue culture dish. The BMM were cultured for 24 hours in macrophage growth medium. BMM were then incubated in macrophage starve medium for 6 hours.

The time-lapse microscope and apparatus were prepared for image recording. The stage was warmed to 37°C. The Dunn chamber was prepared by first washing both inner and outer wells with macrophage starve medium several times. Warm macrophage starve medium was then added to cover both inner and outer wells.

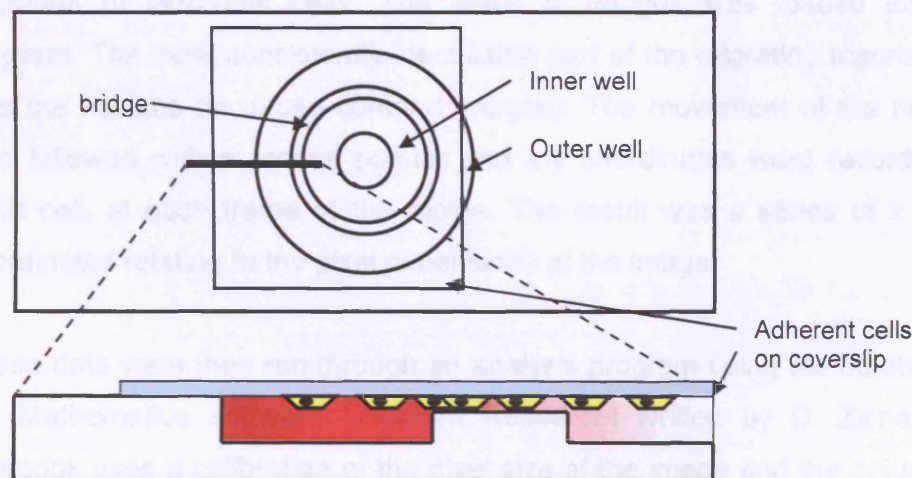


Figure 2.1: The Dunn chemotaxis chamber experimental setup

The coverslip with adherent BMM was then carefully removed from the culture dish and placed over the wells, leaving a small gap on one side of about 2 mm. Excess medium was blotted off using a soft tissue paper and gentle pressure on the coverslip. Hot imaging wax was applied to three sides of the coverslip, sealing three sides to the chamber, and leaving the side with a gap unsealed. With a small piece of filter paper the medium in the outer well was absorbed out through the gap left between coverslip and the outer well. Once the outer well was free of medium, warmed macrophage starve medium containing 33 ng/mL CSF-1 was added using a fine pipette tip (such as a gel

loading tip). This side of the coverslip was then also sealed with the application of hot wax.

In this way, a CSF-1 gradient was formed across the bridge region of the chamber. Cells in the bridge region between the two wells were then observed and phase-contrast images recorded for 18 hours, with frames at 5 minute intervals, using a Zeiss Axiovert 135 microscope, KPM 1E/KS10 CCD Camera and Kinetic Imaging software.

#### 2.3.7: Migration analysis (Motion Tracking)

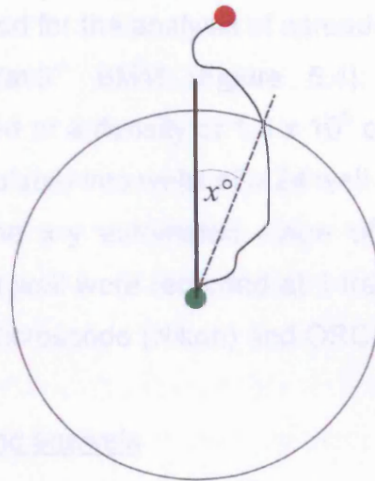
Data were recorded as a series of images (frames) and the Kinetic Imaging motion analysis software or ImageJ tracking software was used to track the migration of individual cells. The stack of images was loaded into the program. The most consistently identifiable part of the migrating macrophage was the nucleus (in phase contrast images). The movement of the nucleus was followed with a mouse pointer and x-y coordinates were recorded for each cell, at each frame of the movie. The result was a series of x and y coordinates relating to the pixel dimensions of the image.

These data were then run through an analysis program using the notebook in the Mathematica software (Wolfram Research) written by D. Zicha. This notebook uses a calibration of the pixel size of the image and the actual size of the image in  $\mu\text{m}$ . This way, coordinates relating to pixels of the image were used to calculate the actual distance between points in the image. With reference to the time between each frame, data such as migration speed and displacement were generated.

#### 2.3.8: Analysis of directionality

This is a brief description of the way in which migration data from chemotaxis experiments was analysed using notebooks written by D. Zicha, for use with the Mathematica program. Individual cell paths were recorded by tracking. The position of a cell when it reached a specified distance (called the horizon)

from its starting position was recorded. This relative position was used to determine the direction of the cell movement.



**Figure 2.2: Determining the direction of cell migration.** Green indicates the starting point of migration, and red, the end-point. The direction in which the cell crosses the horizon distance (grey circle) is taken as its direction of migration, as a relative angle.

This was repeated for all the cells tracked. A Rayleigh test was then carried out, which determines whether there is a significant clustering of these directions. Significant clustering of directions for the population indicated that cells were not moving randomly but in similar directions. If this was migration towards the high concentration of the chemo-attractant, then it was accepted that the population demonstrated positive chemotaxis toward the chemo-attractant.

#### 2.3.9: Spreading analysis by time-lapse microscopy

Detached BMM were resuspended at a density of  $1.6 \times 10^5$  cells/mL in macrophage starve medium. 1 ml volumes of this cell suspension were plated onto 2 cm tissue culture dishes (NUNC or TPP). Image recording by time-lapse phase contrast microscopy was started immediately. Note that at this stage, the cells were not spread, so this image was focussed with reference to the surface of the tissue culture dish. Images were recorded at 1 frame/min

for 2 hours with a Zeiss Axiovert 135 microscope and KPM 1E/KS10 CCD Camera.

This method was modified for the analysis of spreading comparing Wt and the Vav1<sup>-/-</sup>, Vav2<sup>-/-</sup> and Vav3<sup>-/-</sup> BMM (Figure 5.4): As before, cells were resuspended and seeded at a density of  $1.6 \times 10^5$  cell/ml, and 0.5 ml volume of this suspension was plated into wells of a 24 well tissue culture plate (TPP) This was placed on the x-y automated stage of the microscope. Phase contrast images of each well were recorded at 1 frame / min for 2 hours with an Eclipse TE 2000-E Microscope (Nikon) and ORCA-ER CCD Camera.

#### 2.3.10: Cell Morphometric analysis

BMM were resuspended to  $8 \times 10^4$  cells/mL in macrophage growth medium. Cells were seeded onto 2 cm tissue culture dishes and phase-contrast image recording was immediately started. Images were recorded using a x20 objective lens. Images were recorded at 1 frame every 5 minutes for 18 hours with a Zeiss Axiovert 135 microscope and KPM 1E/KS10 CCD Camera.

Movies were recorded as a series of TIF images. These were loaded into the ImageJ image analysis program. Frames from the movies at 1, 5, 9, 13 and 17 hours were retained. Since phase-contrast images were recorded in greyscale, cell boundaries could not be determined by automated methods. Cells boundaries were therefore determined manually. This was an acceptable approach, since in this case it was cell shape that was being analysed as opposed to absolute spread area.

The shape of cells in each of these frames were traced manually. A plug-in for the ImageJ program (circularity- written by Wayne Rasband, NIH), was used to calculate the circularity ratio for each cell in the frame, and an average for the population. Circularity ( $C=4\pi(\text{area}/\text{perimeter}^2)$ ) is a ratio between a shape's area and perimeter, which yields a value of 1 for a perfect circle and approaches 0 as a shape becomes more linear. Thus, the average circularity of cells indicated how the shape of the cells was changed over this time.

### 2.3.11: Immunofluorescent Staining

Medium was aspirated and wells washed with macrophage starve medium warmed to 37°C. Cells were fixed in 3.7% formaldehyde (in PBS with  $Mg^{2+}$  and  $Ca^{2+}$ ) at room temperature for 15 minutes. Coverslips were washed with PBS 3 to 4 times, then, 0.5% Triton in PBS was added to permeabilise the cells for 5 minutes. After extensive washing, blocking solution was added and incubated with the coverslips for 30 minutes at room temperature.

Primary antibody solution was made up in blocking solution at the appropriate concentration. 40  $\mu$ L of this solution were dropped onto parafilm in a 15 cm dish. Inside the lid of the dish, a piece of filter paper was fixed and soaked in water to humidify the container. Coverslips were inverted onto the drops of antibody solution and incubated for 1 hour at room temperature in the humidified container, or overnight at 4°C.

Following this incubation, the coverslips were placed back into 2 mL/well dishes into PBS. Coverslips were washed several times in PBS. Secondary antibody solution was made up in PBS, and TRITC-phalloidin was also added. Fluorophore-containing solutions were protected from light by covering their containers (microfuge tubes, Falcon tubes) with aluminium foil. As before, coverslips were placed on a 40  $\mu$ L drop of the secondary antibody (and TRITC-phalloidin) solution and incubated with humidification for 45 minutes at room temperature.

Coverslips were then washed thoroughly and mounted on glass slides with DAKO mounting medium and allowed to set over-night at 4°C.

### 2.3.12: Confocal Microscopy

A Zeiss LSM Laser-scanning microscope, 40x/1.30 N.A. plan neofluar objective and LSM 510 image recording software were used to observe and record images of the fluorescently stained cells. Images were recorded as

1024 x 1024 pixels, representative of an average of 16 frames. For certain experiments such as quantification of cell spread area, a smaller image size was used (512 x 512), with images representative of 8 frames. Images were exported from the LSM software as 16-bit TIFF images. Microscope settings were not altered during image recording within the same experiment.

#### 2.3.13: Quantification of cell spread area by confocal microscopy

Cells ( $8 \times 10^4$ ) were seeded onto 13 mm diameter round glass coverslips in NUNC 4-well tissue culture dishes, in macrophage starve medium.

The cells were incubated at 37°C, 10% CO<sub>2</sub> for 60 minutes. Non-adherent cells were washed off gently with warm medium. Adherent cells were then fixed with 3.7% formaldehyde in PBS. Fixed cells were permeabilised, blocked, and stained with TRITC-phalloidin as described (section 2.3.11).

Images of fields of cells were recorded using confocal microscopy, selecting fields randomly across the coverslip. TIF images of the F-actin stain only (monochrome) exported from LSM software were loaded in the ImageJ programme.

Images were greyscale. The image 'Threshold' was adjusted to produce a binary image, where any fluorescent signal was given a value of 1 and the background, 0. Thus cells appeared as white shapes on a black background. These shapes were analysed for their pixel area. This was repeated for all the images recorded from a single coverslip. The pooled data were exported to a spread-sheet. Here the data was averaged for each cover slip and tested for significant differences between Wt and Vav-null BMM populations.

#### 2.3.14: Flow Cytometry

BMM were detached in versene-EDTA and added to an equal volume of PBS (without divalent cations). A small aliquot was taken to determine cell density, and the rest of the suspension was centrifuged at 1000 rpm (ALC PK130R)



for 5 minutes. Cells were resuspended in PBS and  $2.5 \times 10^5$  cells were added to round-bottomed polystyrene tubes on ice. Tubes were centrifuged and cells re-suspended in 100  $\mu$ L of FACS buffer (500 mL PBS, 0.1g sodium azide, 10 mL FCS). Fluorophore-conjugated specific or negative control antibody was added to the appropriate concentration (except in the case of non-stained controls). The contents were gently mixed and the tubes were then left on ice for 30 minutes, in the dark.

Cells were then washed using 1 mL volumes of FACS buffer three times. Finally, cells were resuspended in 0.5% paraformaldehyde solution. Samples were read through a flow cytometer (BD FACS Canto flow cytometer), and data analysed by flow cytometry data analysis software (BD FACS Diva and Flowjo).

### **3. The role of Vav proteins in macrophage morphology and migration**

#### **3.1: Introduction**

Migration requires coordination between the regulation of cell morphology and cell adhesion to the surface or matrix. The cell protrudes at the leading edge, extending a lamellipodium that is driven by active actin polymerisation. This protrusion is stabilised by the formation of new adhesions. The cell then contracts behind the nucleus to push it forward. Meanwhile, adhesions at the rear end of the cell are disassembled. In this way, the cell translocates forward (Lauffenburger and Horwitz, 1996).

Cell morphological changes are regulated by the actin and microtubule cytoskeleton. Introduction of constitutively active Rho GTPases into cells has demonstrated their roles in regulating the actin cytoskeleton (Ch. 1.2). However, analysis of blocking the activity of endogenously expressed GTPase can also give insights into their role in regulating cell morphology. For example, expression of V14RhoA (constitutively active) in macrophages induced cell rounding, whereas inhibition of endogenous Rho activity led to cell elongation (Allen et al., 1997). In addition, inhibition of the RhoA effector ROCK led to elongated tails and altered migration of lymphocytes on ICAM-1 (Smith et al., 2003). The GTPase Rac also regulates cell morphology and migration. BMM from Rac-deficient mice have an elongated morphology in culture (Wells et al., 2003; Wheeler et al., 2006). Reduction of Rac1 levels by siRNA in fibroblasts induces cell elongation and increased migration persistence (Pankov et al., 2005). Thus Rho GTPases are regulators of cell morphology and migration.

As Rho/Rac GEFs, Vav proteins have also been implicated in the regulation of cell morphology and migration. The role of Vav proteins in lymphocyte signalling has been extensively studied (reviewed in (Cantrell, 1998; Tybulewicz, 2005)). In polarised T-cells, Vav1 was found to localise to both the leading edge and uropod (Vicente-Manzanares et al., 2005). Furthermore, BMM lacking Vav1 had reduced migration speed (Wells et al., 2005). These

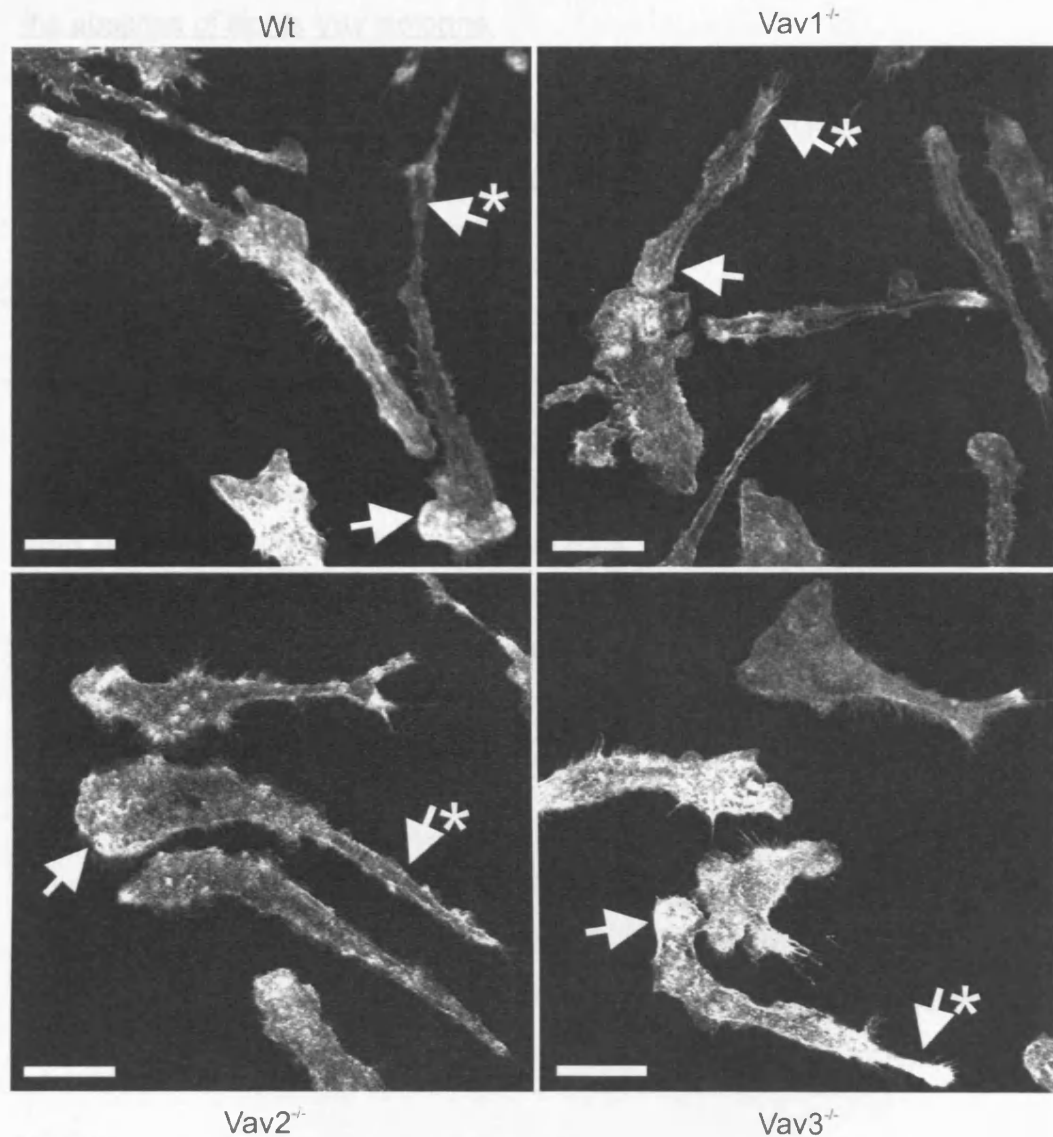


studies highlight the potential of Vav proteins to regulate morphology and migration in leukocytes.

As Vav proteins have been implicated in the regulation of both cell morphology and cell adhesion, the role of Vav proteins in macrophage migration was investigated. Macrophages migrate in normal growing culture conditions (random migration). The migration and morphology of Wt and Vav-deficient BMM cultured in growth medium (containing CSF-1) were therefore compared.

### 3.2: Macrophage morphology is unaffected by the absence of single Vav isoforms

To analyse the effect of the absence of Vav proteins on macrophage morphology, the actin cytoskeleton was visualised in Wt, Vav1<sup>-/-</sup>, Vav2<sup>-/-</sup> and Vav3<sup>-/-</sup> BMM (Figure 3.1). The primary macrophage populations were observed to be very heterogeneous. Within the same population cells adopted a number of different morphologies and there were also subtle differences between experiments. Significant variations in morphology were therefore difficult to determine by observation. In general however, macrophages cultured in growth medium adopted a migratory morphology, consisting of a clearly defined leading edge (arrows) and tail (starred arrows). F-actin was concentrated at the leading edge of some cells, and also at the extreme edge of tail structures. F-actin puncta were also localised in the leading edge of some cells, indicating the presence of podosomes (Wheeler et al., 2006). No clear morphological differences were consistently observed between Wt and Vav-null BMM. Though morphologies were not quantitated, the loss of single isoforms of Vav did not appear to significantly affect macrophage morphology.



**Figure 3.1: BMM morphology is unaffected by the absence of single Vav isoforms.** Wt, Vav1<sup>-/-</sup>, Vav2<sup>-/-</sup> and Vav3<sup>-/-</sup> BMM were cultured on glass coverslips in growth medium for 24 hours. Cells were then fixed and stained for F-actin with TRITC-phalloidin. Bar=20  $\mu$ m. Arrows: leading edge. Starred arrows: tail structures. Images were acquired by laser scanning confocal microscopy. Images are representative of three independent experiments.

### 3.3: BMM migration on tissue culture plastic is not significantly affected by the absence of single Vav isoforms.

To determine whether loss of single Vav proteins affected macrophage migration, Wt, Vav1<sup>-/-</sup>, Vav2<sup>-/-</sup> and Vav3<sup>-/-</sup> BMM were recorded for 18 hours by time-lapse phase-contrast microscopy.

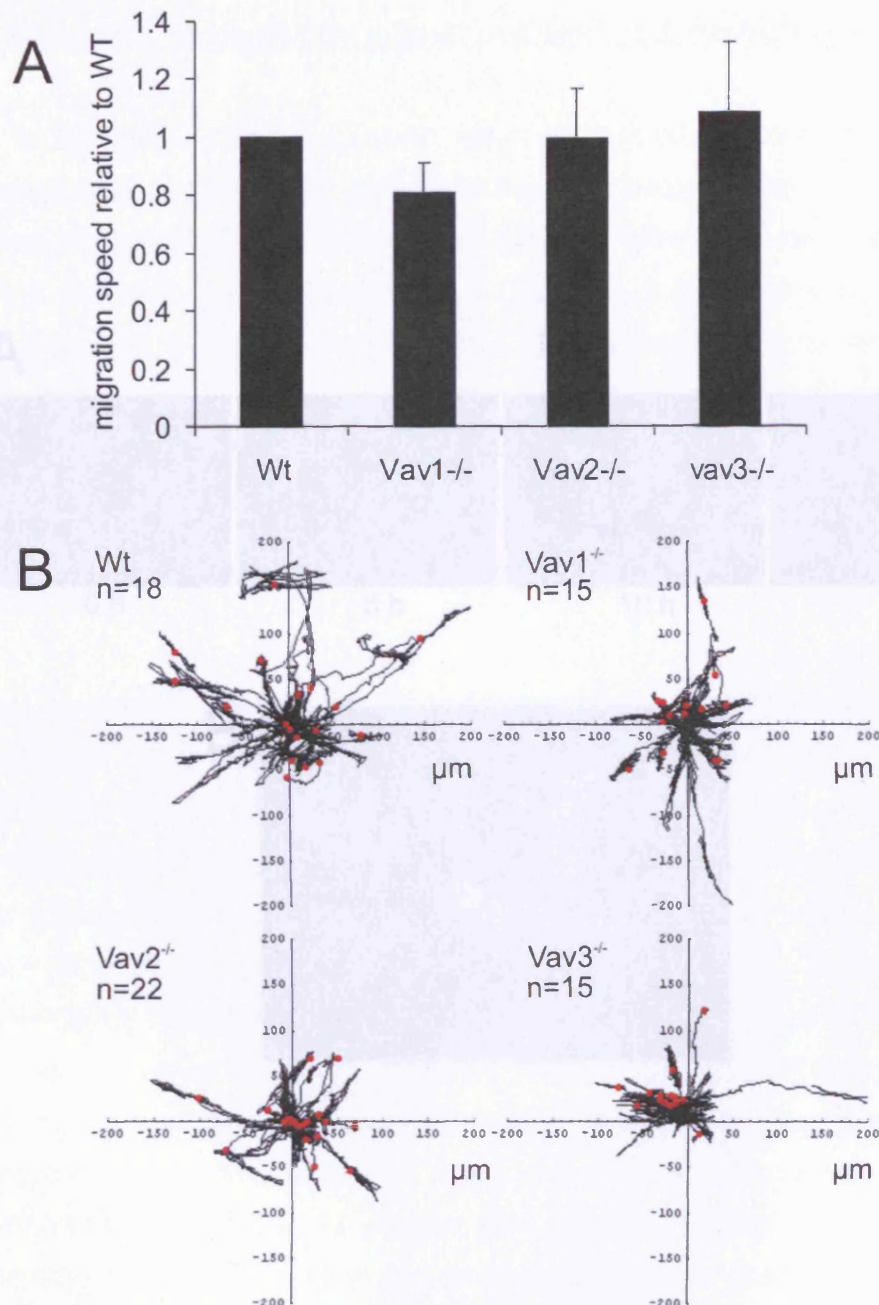
Vav1<sup>-/-</sup> BMM had reduced migration speed compared to Wt BMM (Figure 3.2A), as reported previously (Wells et al., 2005). Vav2<sup>-/-</sup> BMM had a similar speed to Wt BMM, whereas BMM lacking Vav3 had a slightly increased speed. However, due to the variability between experiments these differences were not statistically significant. Figure 3.2B shows representative tracks of the movies recorded for each population of BMM. These diagrams show the paths of individual cells with the origins centred. Over three experiments, consistent differences in migration between Wt and BMM lacking single isoforms of Vav were not observed.

	Wt	Vav1 <sup>-/-</sup>	Vav2 <sup>-/-</sup>	Vav3 <sup>-/-</sup>
Mean speed $\pm$ s.e.m ( $\mu\text{m}/\text{min}$ )	0.65 $\pm$ 0.06	0.64 $\pm$ 0.10	0.45 $\pm$ 0.02	0.70 $\pm$ 0.10

**Table 3.1: Wt and Vav-null BMM migration speeds on tissue culture plastic**

Migration speeds of the Wt and Vav-null BMM were comparable to previous studies (Wells et al., 2004; Wheeler et al., 2006). Most notably, cells translocated very little. Cells extended at the front, but did not retract their tails (Figure 3.3A and movie 5). Migration speed was largely attributed to movement of the nucleus along the long axis of the cell: the nucleus shuttled back and forth, with little cell translocation (Figure 3.3B). Recorded speeds are not therefore representative of cell translocation.

Thus, no differences were observed in the migration of macrophages lacking single isoforms of Vav, however the experimental conditions may not have been optimal for detecting differences in macrophage migration.

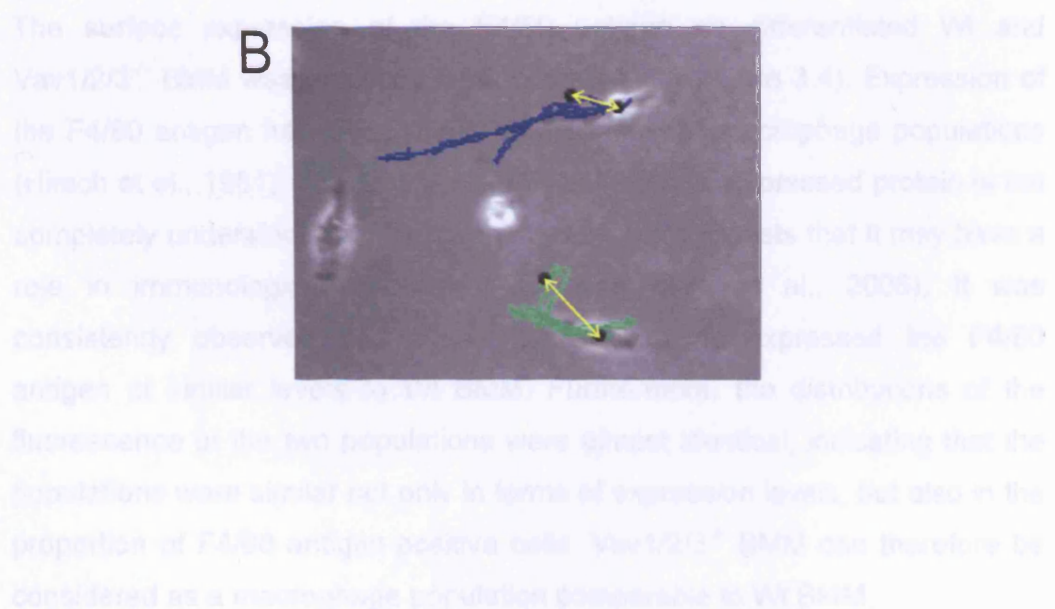
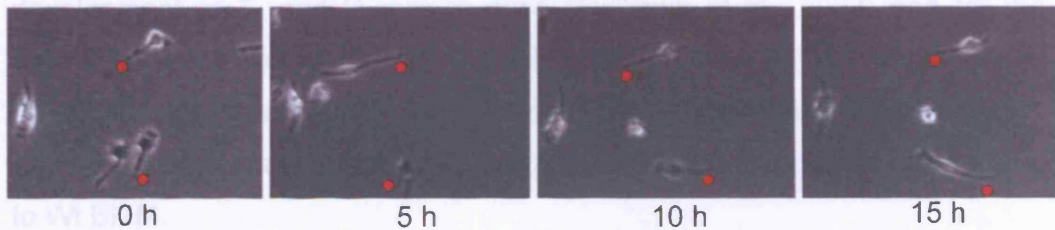


**Figure 3.2: BMM migration on tissue culture plastic is not significantly affected by the absence of single Vav isoforms.** Wt, Vav1<sup>-/-</sup>, Vav2<sup>-/-</sup> and Vav3<sup>-/-</sup> BMM were allowed to adhere to plastic dishes in growth medium for 24 hours. Cells were then observed by time-lapse phase-contrast microscopy for 18 hours, acquiring images at 1 frame/5 min (movies 1, 2, 3 and 4). Individual cell tracks (n≥15 per experiment) were followed using tracking software, and data analysed using migration analysis software (see Ch. 2.3). (A) shows the mean migration speed ±s.e.m. for three independent experiments and (B) shows the migration paths of cells with their origins centred, from a representative experiment (Red indicates the end-point of migration).

### 3.4 *Vav1/2/3*<sup>-/-</sup> bone-marrow cells differentiate into a macrophage population

It is possible that Vav proteins have redundant functions in regulating morphology and migration. Cells were therefore isolated from mice lacking all three isoforms of Vav. However, Vav proteins have been reported to effect gene transcription in lymphocytes (Cantrek, 1999; Tybulewicz, 2003).

Furthermore, it has been shown that Vav proteins are essential for the



**Figure 3.3: Failure of tail retraction may contribute to low BMM translocation.**

Wt BMM were seeded in growth medium on tissue culture plastic dishes for 24 hours. BMM were then filmed by time-lapse phase-contrast microscopy for 16 hours (movie 5). (A) shows frames of the movie at the indicated timepoints. Red circles marks the tail of the cell. (B) Cell migration was recorded by tracking. Black circles mark the start and end points. Yellow arrows indicate the cell's displacement during the filming.

3.4: Vav1/2/3<sup>-/-</sup> bone-marrow cells differentiate into a macrophage population.

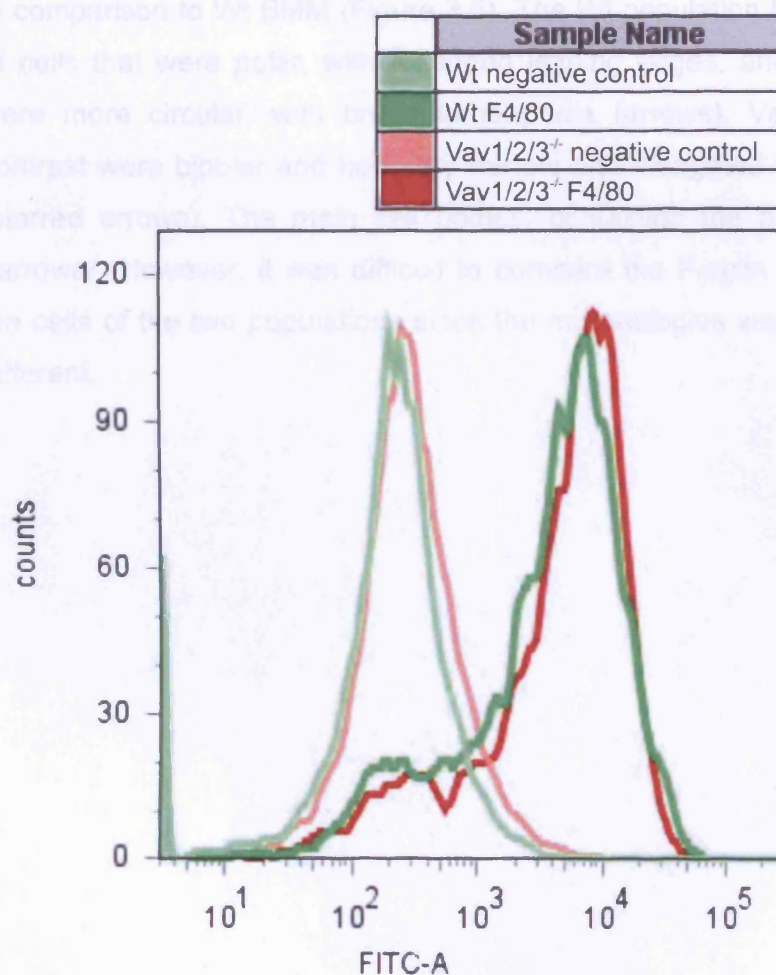
It is possible that Vav proteins have redundant functions in regulating morphology and migration. Cells were therefore isolated from mice lacking all three isoforms of Vav. However, Vav proteins have been reported to affect gene transcription in lymphocytes (Cantrell, 1998; Tybulewicz, 2005). Furthermore, it has been shown that Vav proteins are essential for the development of T- and B-cells in mice (Fujikawa et al., 2003) and for the neutrophil-like differentiation of myeloid precursor cells (Bertagnolo et al., 2004). It was therefore important to determine whether cells derived from Vav1/2/3<sup>-/-</sup> mice could differentiate into a macrophage population comparable to Wt BMM.

The surface expression of the F4/80 antigen on differentiated Wt and Vav1/2/3<sup>-/-</sup> BMM was analysed by flow cytometry (Figure 3.4). Expression of the F4/80 antigen has been shown to be limited to macrophage populations (Hirsch et al., 1981). The function of this cell-surface expressed protein is not completely understood, although recent research suggests that it may have a role in immunological tolerance (van den Berg et al., 2005). It was consistently observed that Vav1/2/3<sup>-/-</sup> populations expressed the F4/80 antigen at similar levels to Wt BMM. Furthermore, the distributions of the fluorescence of the two populations were almost identical, indicating that the populations were similar not only in terms of expression levels, but also in the proportion of F4/80 antigen-positive cells. Vav1/2/3<sup>-/-</sup> BMM can therefore be considered as a macrophage population comparable to Wt BMM.



### 3.5: Vav1/2/3<sup>-/-</sup> BMM have an elongated morphology in culture.

The effect of the loss of all three isoforms of Vav on macrophage morphology was then analysed by staining air-dried fragments of Wt and Vav1/2/3<sup>-/-</sup> BMM. When cultured on glass Vav1/2/3<sup>-/-</sup> BMM exhibited an elongated morphology in comparison to Wt BMM (Figure 3.4). The Wt cells were typically composed of cells that were polar, with some cells that were more circular, with bipolar and hexapolar cells. The Vav1/2/3<sup>-/-</sup> BMM in contrast were bipolar and hexapolar. The cells were also stained with F4/80 antibody. However, it was difficult to compare the F4/80 expression between the cells of the two populations as the morphologies were so dramatically different.



**Figure 3.4: Vav1/2/3<sup>-/-</sup> BMM F4/80 antigen expression is comparable to Wt BMM.** Equal numbers of Wt and Vav1/2/3<sup>-/-</sup> BMM were resuspended in FACS buffer. Cells were then incubated on ice with either FITC-conjugated F4/80 antibody or an isotype specific negative control for 30 minutes. Cells were washed and then analysed for expression of the F4/80 antigen by flow cytometry. Data are representative of three independent experiments.

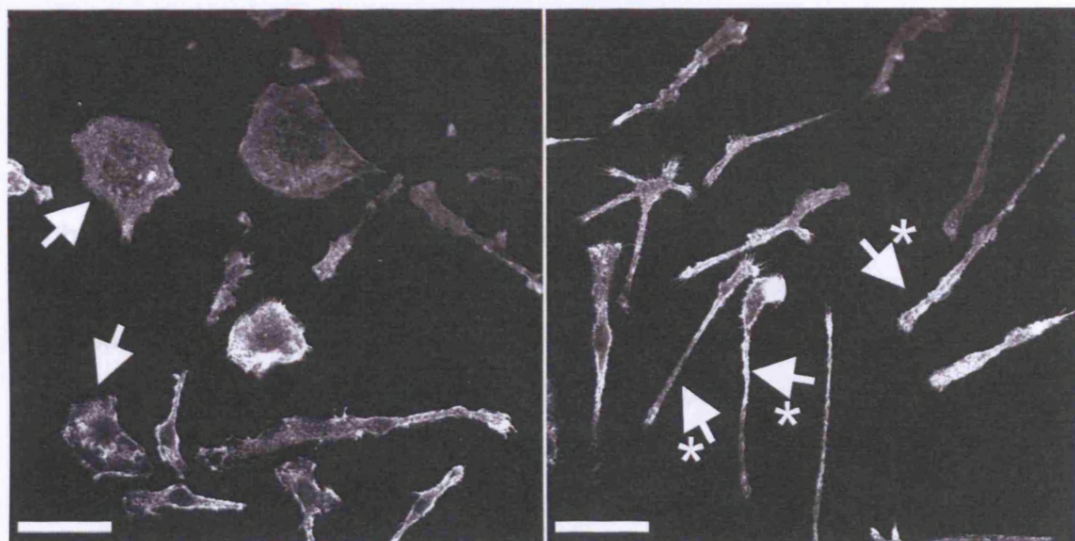
### 3.5: Vav1/2/3<sup>-/-</sup> BMM have an elongated morphology in culture.

The effect of the loss of all three isoforms of Vav on macrophage morphology was then analysed by staining actin filaments of Wt and Vav1/2/3<sup>-/-</sup> BMM. When cultured on glass Vav1/2/3<sup>-/-</sup> BMM exhibited an elongated morphology in comparison to Wt BMM (Figure 3.5). The Wt population typically consisted of cells that were polar, with extended leading edges, and some cells that were more circular, with broad lamellipodia (arrows). Vav1/2/3<sup>-/-</sup> BMM in contrast were bipolar and had very narrow and elongated lamellae and tails (starred arrows). The main cell bodies, containing the nucleus were also narrower. However, it was difficult to compare the F-actin distribution within the cells of the two populations since the morphologies were so dramatically different.



### 3.6 The elongated morphology of $Vav1/2/3^{-/-}$ BMM develops independently of chemokine attraction

To investigate how the elongated morphology of  $Vav1/2/3^{-/-}$  BMM developed, growing Wt and  $Vav1/2/3^{-/-}$  BMM were re-seeded onto culture dishes in chemokine growth medium (containing CSF-1), and monitored over 24 hours as they spread in normal growing conditions (Figure 3.5 and movies 3 and 7). This time we simulated the conditions in which the



$Vav1/2/3^{-/-}$  BMM were more widely purified. Interestingly, some elongated cells were observed in dishes in which no chemokine was present. The elongated morphology of  $Vav1/2/3^{-/-}$  BMM observed in dishes in which no chemokine was present was therefore not transient but generally developed and maintained over 24 hours. Interestingly, it appeared that elongated cells had tail retraction indicating the contribution of the phenotype.

Changes in cell shape over time were analyzed in Wt and  $Vav1/2/3^{-/-}$  BMM by analyzing cells in selected frames of the 24-hour movie (Figure 3.7).

Cell shapes were analyzed by their geometry, which is a ratio of a shape's

**Figure 3.5:  $Vav1/2/3^{-/-}$  BMM adopt an elongated morphology in culture.** Wt and  $Vav1/2/3^{-/-}$  BMM were cultured on glass coverslips in growth medium for 24 hours. Cells were then fixed and stained for F-actin with TRITC-phalloidin. Images were acquired by laser scanning confocal microscopy. Images are representative of three independent experiments. Arrows: Lamellipodia. Starred arrows: elongated tails and leading edges. Bar = 40  $\mu$ m.

### 3.6: The elongated morphology of Vav1/2/3<sup>-/-</sup> BMM develops progressively upon adhesion.

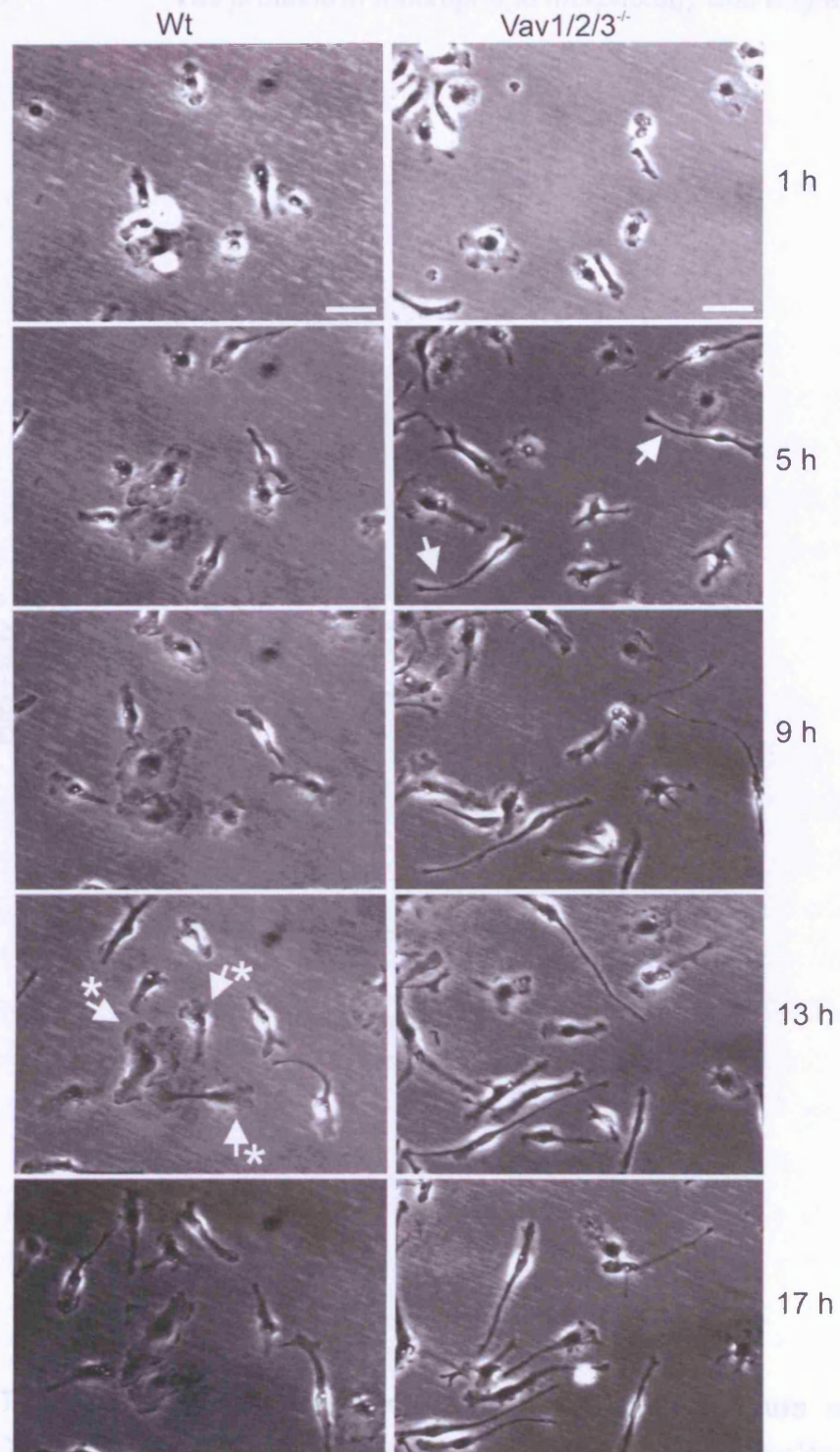
To investigate how the elongated morphology of Vav1/2/3<sup>-/-</sup> BMM developed, growing Wt and Vav1/2/3<sup>-/-</sup> BMM were re-seeded onto culture dishes in macrophage growth medium (containing CSF-1), and monitored over 24 hours as they spread in normal growing conditions (Figure 3.6 and movies 6 and 7). This therefore simulated the conditions of normal culture in which the two BMM populations adopted differing morphologies.

Immediately after seeding, cells were very bright in the phase contrast images, indicating a spherical shape (Figure 3.6). Within the first hour of adhesion, Wt and Vav1/2/3<sup>-/-</sup> populations spread at similar rates and both populations consisted of either circular or polarised cells actively ruffling and extending lamellipodia. By 5 hours, Vav1/2/3<sup>-/-</sup> cells became clearly elongated in comparison to Wt, most notably by the appearance of elongated tails (arrows). Wt BMM maintained a mixed population of flat spread or polarised (migratory) cells with broad lamellae and comparatively short tails even at later time-points (relative to Vav1/2/3<sup>-/-</sup> BMM) (starred arrows), whereas Vav1/2/3<sup>-/-</sup> BMM were more uniformly polarised. Interestingly, some elongated Vav1/2/3<sup>-/-</sup> BMM had elongated leading edges as well as elongated tails. The elongated morphology of Vav1/2/3<sup>-/-</sup> BMM observed in static images was therefore, not transient but gradually developed and maintained over 3-4 hours. Interestingly, it appeared that abnormal protrusion and tail retraction contributed to the phenotype.

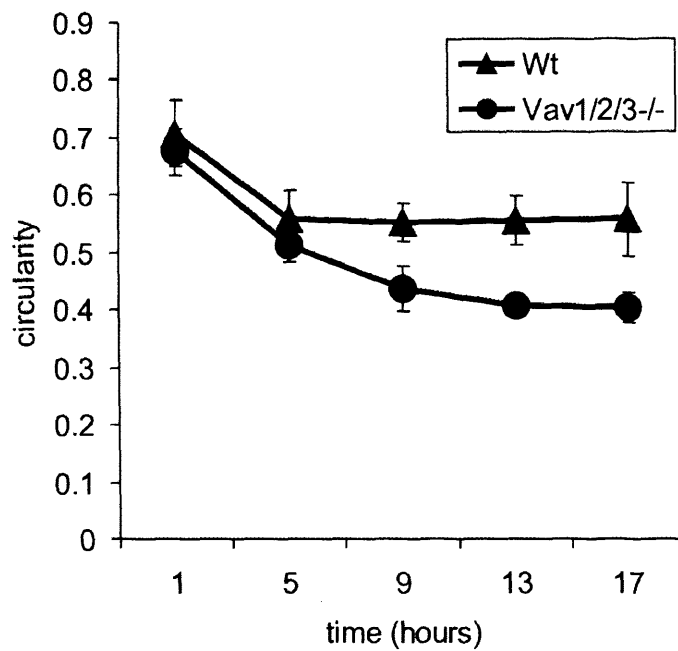
Changes in cell shape over time were followed for both Wt and Vav1/2/3<sup>-/-</sup> BMM by analysing cells in selected frames of the 18-hour movie (Figure 3.7). Cell shapes were analysed for their circularity, which is a ratio of a shape's area to its perimeter. This value is 1 for a perfect circle, and approaches 0 as shapes become more elongated, or linear.

From 1 to 5 hours both populations had similar circularity during spreading. At 1 hour, a high circularity ratio of ~0.7 was recorded for both populations as

they spread as circular, flat cells. By 5 hours, cells had become more bipolar, and the circularity decreased to 0.55 (Wt) and 0.5 (Vav1/2/3<sup>-/-</sup>). After this time the Wt population maintained a mean circularity of 0.55, indicating that the proportion of polarised cells and circular cells remained similar. In contrast Vav1/2/3<sup>-/-</sup> BMM became less circular over time, with a circularity ratio of 0.4 at 17 hours. This indicates that the major change in morphology occurs after 5 hours of spreading. Thus the elongated morphology of macrophages lacking Vav proteins does not develop immediately after adhesion, but once cells begin to polarise.



**Figure 3.6: Vav1/2/3<sup>-/-</sup> BMM elongated morphology develops 3/4 hours after adhesion.** Wt and Vav1/2/3<sup>-/-</sup> BMM were resuspended in growth medium and seeded onto tissue culture dishes. Cells were observed by time-lapse phase-contrast microscopy, acquiring images for 18 hours at 1 frame/5 min (movies 6 and 7). Arrows: elongated tails. Starred arrows: broad protrusions. Bar=40 μm. Shown are frames from a representative movie of three independent experiments.



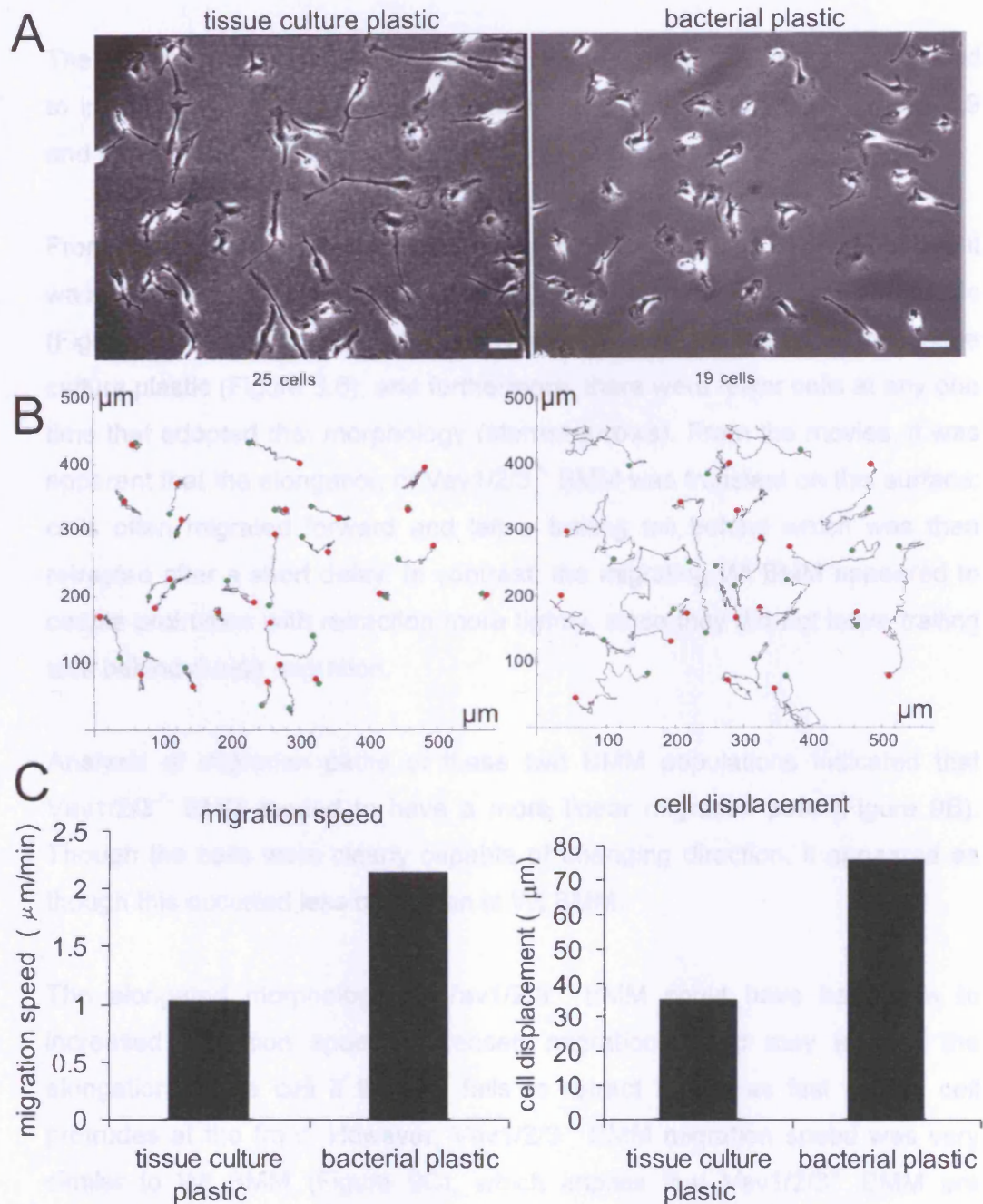
**Figure 3.7: Vav1/2/3<sup>-/-</sup> BMM elongation is apparent from 5 hours after seeding.** Cell 'circularity' was compared at different times after seeding for Wt and Vav1/2/3<sup>-/-</sup> BMM. Phase contrast images at the indicated times after seeding were analysed for cell shape using an image analysis program. Cell outlines in each frame ( $n > 15$ , for all experiments) were analysed for their circularity (circularity =  $4\pi(\text{area}/\text{perimeter}^2)$ ), which gives a ratio from 0 (circular) to 1 (linear). Shown here are the mean circularity values for each time point for three independent experiments  $\pm$  s.e.m.



### 3.7: BMM migration is greatly enhanced on bacterial plastic

It was possible that the low migration speed and lack of translocation observed on tissue culture plastic was due to the strong adhesiveness of macrophages on this surface. In general, cells do not adhere to untreated plastic. Tissue culture plastic is chemically treated to generate ionised reactive groups in an otherwise unreactive polymer. Bacterial plastic is an untreated surface used to sub-culture macrophages, a surface on which they are adherent, but less so than on tissue culture plastic. The migration of macrophages plated on tissue culture plastic was therefore compared with that on bacterial plastic (Figure 3.8 and movies 8 and 9).

Analysis of the migration of macrophages on the two surfaces revealed that migration speed was greater on bacterial plastic (Figure 3.8C). Cell displacement was also increased on bacterial plastic (Figure 3.8B and C), indicating increased cell translocation. Phase contrast images of migrating macrophages depict the different morphologies adopted on the two culture surfaces (Figure 3.8A). Interestingly, on tissue culture plastic, macrophages adopt a 'migratory' morphology as previously observed (movies 1 to 4). These macrophages had broad lamellae, and thin trailing tails. However, this morphology failed to produce much net cell translocation. Macrophages on bacterial plastic appeared much less spread and elongated. Lamellae were less elongated but appeared to encompass more of the cell perimeter. Overall, on bacterial plastic, cells occupied a smaller spread area, and trailing tails of macrophages appeared shorter, and in some cases could not be distinguished from the main body of the cell.



**Figure 3.8: BMM have increased migration on bacterial plastic compared to tissue culture plastic.** Equal numbers of Wt BMM were cultured on tissue culture plastic dishes or bacterial plastic for 24 hours in growth medium. Cells were then observed by time-lapse phase-contrast microscopy for 3 hours acquiring 1 frame/min (movies 8 and 9) (A). Individual cell tracks were then recorded using tracking software (green: start, red: end-point) (B) and analysed using migration analysis software (see Ch. 2.3), to compare migration speed and cell displacement (C).

### 3.8 Vav1/2/3<sup>-/-</sup> BMM are more persistent during migration

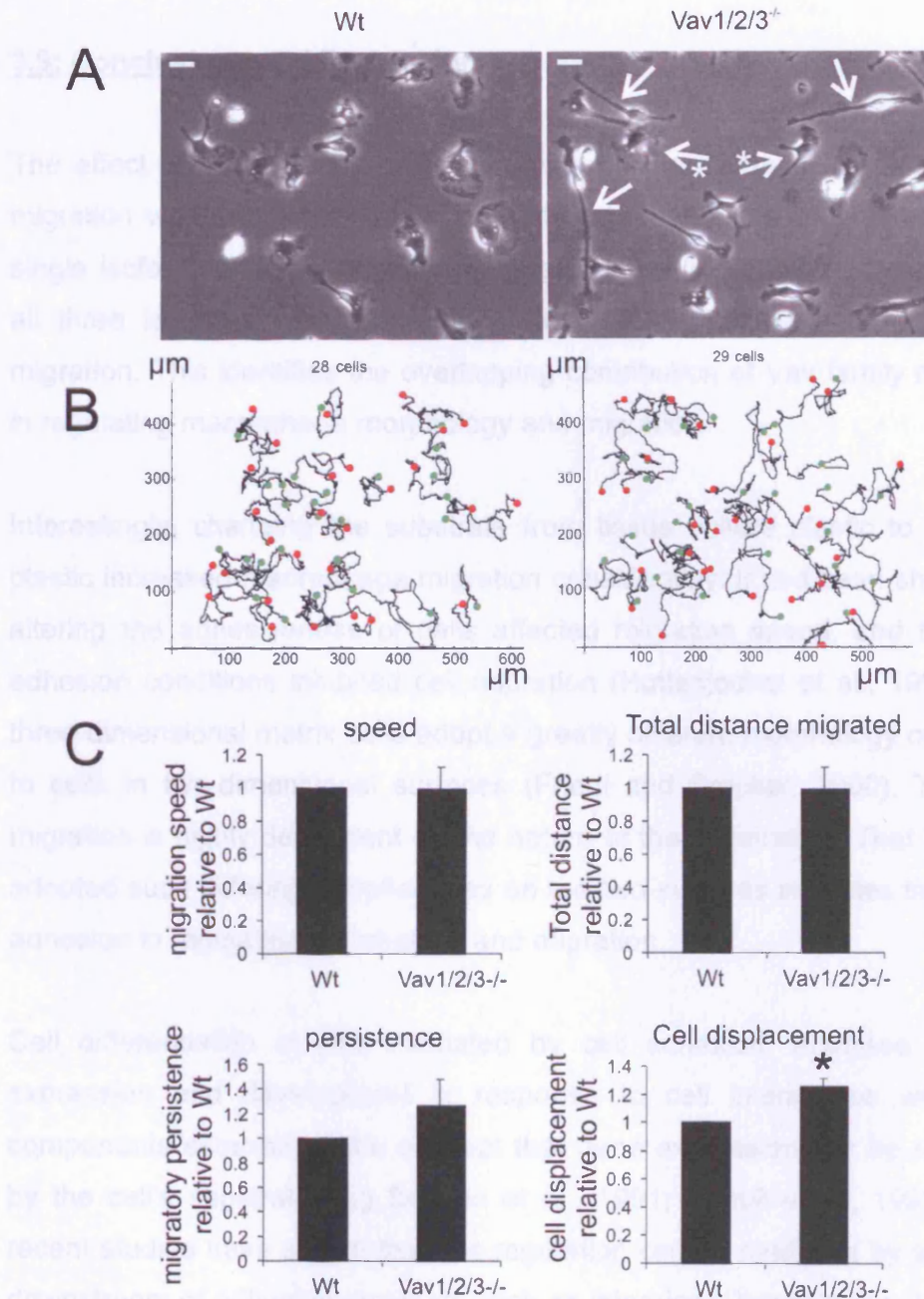
The migration of Wt and Vav1/2/3<sup>-/-</sup> BMM on bacterial plastic was compared to investigate the role of Vav proteins in macrophage migration (Figure 3.9 and movies 10 and 11).

From observation of phase contrast images of the Vav1/2/3<sup>-/-</sup> macrophages it was clear that their elongated morphology was retained upon bacterial plastic (Figure 3.9A) (arrows). However they were not as elongated as on tissue culture plastic (Figure 3.6), and furthermore, there were fewer cells at any one time that adopted that morphology (starred arrows). From the movies, it was apparent that the elongation of Vav1/2/3<sup>-/-</sup> BMM was transient on this surface: cells often migrated forward and left a trailing tail behind which was then retracted after a short delay. In contrast, the migrating Wt BMM appeared to couple protrusion with retraction more tightly, since they did not leave trailing tails behind during migration.

Analysis of migration paths of these two BMM populations indicated that Vav1/2/3<sup>-/-</sup> BMM tended to have a more linear migration path (Figure 9B). Though the cells were clearly capable of changing direction, it appeared as though this occurred less often than in Wt BMM.

The elongated morphology of Vav1/2/3<sup>-/-</sup> BMM could have been due to increased migration speed. Increased migration speed may lead to the elongation of the cell if the cell fails to retract its tail as fast as the cell protrudes at the front. However, Vav1/2/3<sup>-/-</sup> BMM migration speed was very similar to Wt BMM (Figure 9C), which implies that Vav1/2/3<sup>-/-</sup> BMM are defective in tail retraction. There was no difference in the total distance migrated by each cell population. However, Vav1/2/3<sup>-/-</sup> BMM were displaced a significantly greater distance (from starting point to the end point of migration), which was reflected in an increased persistence of migration. Vav1/2/3<sup>-/-</sup> BMM therefore migrate at a similar speed to Wt BMM, however, they migrate more persistently, which results in greater cell displacement





**Figure 3.9: Vav1/2/3<sup>-/-</sup> BMM have enhanced migration persistence and greater cell displacement.** Wt, Vav1/2/3<sup>-/-</sup> BMM were allowed to adhere to bacterial plastic dishes in growth medium for 24 hours. Cells were then observed by time-lapse phase-contrast microscopy for 3 hours, acquiring images at 1 frame/min (A)(movies 10 and 11). Arrows: elongated cells. Starred arrows: normal cells. Individual cell tracks ( $n > 15$  for each experiment) were followed using tracking software, and data analysed using migration analysis software (green: start, red: end-point) (B). (A) and (B) are a representative experiment of three independent experiments. (C) shows the mean migration speed, total distance migrated, persistence and cell displacement from the origin relative to Wt  $\pm$  s.e.m. for three independent experiments. Statistical significance was determined by a Student's *t* test (\*  $P < 0.05$ ). Bar = 20  $\mu$ m.

### **3.9: Conclusions and Discussion**

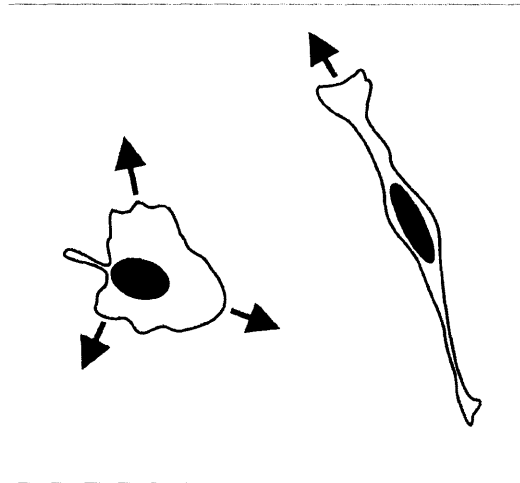
The effect of the absence of Vav proteins on macrophage morphology and migration was studied. It was observed that whereas macrophages lacking single isoforms of Vav had a normal macrophage morphology, those lacking all three isoforms were abnormally elongated in culture and had altered migration. This identifies the overlapping contribution of Vav family members in regulating macrophage morphology and migration.

Interestingly, changing the substrate from tissue culture plastic to bacterial plastic increased macrophage migration considerably. It had been shown that altering the adhesiveness of cells affected migration speed, and that high adhesion conditions inhibited cell migration (Huttenlocher et al., 1996). In a three-dimensional matrix cells adopt a greatly different morphology compared to cells in two-dimensional surfaces (Friedl and Brocker, 2000). Thus cell migration is highly dependent on the nature of the substratum. That Wt BMM adopted such differing morphologies on the two surfaces indicates the role of adhesion in regulating morphology and migration.

Cell differentiation is also mediated by cell adhesion. Analyses of gene expression and development in response to cell interactions with ECM components established the concept that gene expression can be regulated by the cell's substrate (e.g Benson et al., 1991; Streuli et al., 1993). More recent studies have shown that this regulation can be mediated by signalling downstream of adhesion receptors such as integrins (Giancotti and Ruoslahti, 1999). It is therefore possible that initial adhesion during the differentiation of bone marrow cells determines the level of expression of certain genes and optimises the cell for migration on that particular substrate. BMM may be more motile on bacterial plastic because that is the substrate on which they are differentiated. It would therefore be interesting to compare gene expression on the two different substrata.

Despite their elongated morphology, *Vav1/2/3<sup>-/-</sup>* BMM had a similar migration speed to Wt BMM on bacterial plastic. *Vav1/2/3<sup>-/-</sup>* macrophages did however,

have increased cell displacement and migration persistence. This is likely to be related to the morphology of these cells. In maintaining a more linear shape, *Vav1/2/3*<sup>-/-</sup> macrophages may be less able to change direction, resulting in a more linear trajectory, whereas Wt macrophages, having a broader leading edge and shorter tail would be more prone to turning (Figure 3.10).



**Figure 3.10. Effects of morphology on migration.** A cell with a broad lamellipodium may frequently change direction, as any part of the leading edge may become dominant. An elongated cell has a comparatively narrow lamellipodium, and so is limited in the directions it can protrude into.

As Rac GEFs, *Vav1/2/3*<sup>-/-</sup> BMM could have an altered morphology due to abnormal activation of this GTPase. Loss of Rac activity in *Rac1*- and *Rac1/2*-deficient BMM resulted in an elongation of these cells (Wells et al., 2003; Wheeler et al., 2006). *Rac2*-deficient haemopoietic stem cell progenitors have elongated cell protrusions (filopodia) and show increased migration toward SDF-1 (Yang et al., 2001). However, it was observed that the level of *Rac1* activity was not different in Wt and *Vav1/2/3*<sup>-/-</sup> BMM cultured in growth medium (Chapter 4). It is possible that the absence of Vav proteins only causes a small alteration in the total level of Rac activation, due to the presence of other GEFs that can also act on Rac (reviewed (Rossman et al., 2005)). The major role Vav proteins could be to affect Rac activity in a

specific localisation, rather than its magnitude. Even so, it has been demonstrated that small reductions in Rac activity may cause significant alteration of morphology and migration properties. SiRNA knock-down of Rac resulting in a 30% reduction of Rac activity altered primary fibroblast morphology, making cells more elongated and significantly more persistent during migration (Pankov et al., 2005).

These results demonstrate that often the loss of a protein or function lead to some enhanced property in vitro. For example, Neutrophils deficient in the Rho GEF Lsc had increased migration speed (though with reduced directionality) (Francis et al., 2006). *Vav1/2/3<sup>-/-</sup>* BMM had an elongated morphology and increased cell displacement during migration. Regulators of cell morphology such as Rac and RhoA, have been shown to contribute to cell migration. However the loss of one of these components does not necessarily produce a negative effect on all aspects of cell migration in vitro. In addition these altered migration properties in vitro may not represent the migration in a three-dimensional setting or in vivo. It would therefore be interesting to investigate how two-dimensional migration of macrophages in vitro relates to migration in more physiological conditions.

Cell elongation became apparent after several hours of spreading on tissue culture plastic. The change in morphology may therefore be a result of a defect in adhesion withdrawal, which manifests when cells begin to migrate, but fail to retract their tails. Interestingly, *Vav1/2/3<sup>-/-</sup>* BMM did not appear to be elongated to the same extent on bacterial plastic, on which BMM appear to be less adherent. There is substantial evidence linking Vav proteins to adhesion-dependent signalling in leukocytes (Chapter 5). In migrating cells adhesions need to be differentially regulated in the front and rear of the cell. FAK (and Pyk2 in leukocytes) is important to adhesion signalling, but it has also been shown to be required for adhesion turnover in keratinocytes (Schober et al., 2007). The protein tyrosine phosphatase PTP-PEST was shown to associate with and dephosphorylate paxillin, a scaffold protein essential to integrin signalling (Shen et al., 2000). Furthermore, PTP-PEST-null fibroblasts displayed increased focal adhesion size and reduced migration (Angers-

Loustau et al., 1999). Therefore, proper signalling at adhesion sites regulates their formation, maintenance and disassembly appropriately. Defective signalling at adhesions due to absence of Vav proteins could, therefore, lead to cell elongation.

Vav proteins may be required for de-adhesion due to their activity as Rho/Rac GEFs. The role of Rho family GTPases in the regulation of the actin cytoskeleton has been well-studied. However, evidence also suggests that they have important roles in adhesion disassembly. For example, in migrating T-cells, detachment of the uropod was shown to require the activity of the RhoA effector ROCK (Smith et al., 2003). In this study, treatment of migrating cells with a ROCK inhibitor resulted in elongated tail structures, but also more linear migration. Interestingly, the authors suggested that as well as regulating uropod contraction, the RhoA/ROCK pathway may also regulate adhesion stability. RhoA/ROCK signalling is also important in monocyte migration, where it negatively regulates integrin signalling, allowing controlled cell protrusion and thus, efficient cell migration (Worthylake and Burridge, 2003). Furthermore, in rat glioma cells, a pathway involving RhoA and its effector mDia, was shown to be required for adhesion turnover and migration (Yamana et al., 2006).

Signalling through the Rho GTPases regulates both the actin cytoskeleton and the microtubule network. Rho activity has been shown to promote microtubule stabilisation (Cook et al., 1998). The mechanism of this regulation was later shown to involve the RhoA effector mDia (Palazzo et al., 2001). The GTPase Rac1 promotes microtubule growth (Wittman et al., 2003), possibly by phosphorylation of Stathmin/Op18 microtubule-destabilising protein catalysed by the Rac/Cdc42 effector PAK1 (Daub et al., 2001), though other mechanisms may also exist.

Microtubule ends can associate with adhesion sites in fibroblasts (Kaverina et al., 1998), and disruption of the microtubule network caused increased stability of cell adhesion complexes (Kaverina et al., 1999). In migrating melanoblasts microtubule disruption resulted in elongated tail structures and

an inhibition of cell migration (Ballestrem et al., 2000). These studies demonstrate the important contribution of microtubules to regulation of adhesion turnover. Vav proteins by their activity as GEFs for Rho family GTPases could regulate cell adhesion turnover in migrating cells, and therefore, macrophage morphology.

BMM elongation could also be a result of altered gene expression caused by the absence of Vav proteins. Bone-marrow cells may fail to differentiate into a normal macrophage population, and therefore exhibit a different morphology in culture. There is evidence to suggest that Vav proteins could regulate gene expression and differentiation. Vav1 is the Vav family member that is largely restricted to cells of haemopoietic lineage (Bustelo, 2000). This therefore suggests that it may be required for regulating differentiation in haemopoietic sub-populations. Vav1 was shown to be required for all-trans retinoic acid-induced granulocytic differentiation of the HL-60 myeloid leukaemia cell line (Bertagnolo et al., 2004). Furthermore, Vav1 has been shown to be a component of transcriptionally active complexes in the nucleus including NFAT in mast cells (Houlard et al, 2002). In addition, mice lacking all three Vav proteins fail to develop normal T- and B-cell populations (Fujikawa et al., 2003). However, Vav1/2/3<sup>-/-</sup> cells express the macrophage-specific antigen, F4/80 to the same extent as Wt BMM. Vav proteins are therefore not required for bone-marrow cell differentiation into macrophages. This also shows that the altered morphology of Vav1/2/3<sup>-/-</sup> BMM was not due to abnormal differentiation.

It is still possible that though Vav1/2/3<sup>-/-</sup> have differentiated properly into macrophages, the expression of certain genes that are important in cell morphological regulation are differentially expressed. Interestingly, the elongated shape of Vav1/2/3<sup>-/-</sup> macrophages became apparent a number of hours after seeding onto the dish. The timecourse of the morphological changes do suggest that altered protein expression could be involved, however, a proteomic or gene expression study would have to be conducted to investigate this possibility further.

In summary, Vav proteins have redundant functions in maintaining normal morphology and migration in macrophages. Macrophages lacking Vav proteins adopt an elongated morphology and migrate more persistently during migration. However, further study is required to determine how Vav proteins are involved in this behaviour, and how these altered migration properties relate to macrophage migration in vivo.

#### **4. The role of Vav proteins in CSF-1-induced macrophage morphological responses and chemotaxis**

##### **4.1: Introduction**

Colony stimulating factor is a macrophage chemo-attractant (Webb et al., 1996). In cultured macrophages CSF-1 stimulation induces morphological changes including dorsal membrane ruffles and protrusion of lamellipodia leading to cell spreading. These morphological changes are driven by actin polymerisation (Hall, 1998). The actin cytoskeleton can be organised in different ways to regulate cell morphology. RhoA, Rac and Cdc42 have been shown to regulate the cytoskeleton in macrophages (Allen et al., 1997). In particular, Rac1 mediates the formation of membrane ruffles and lamellipodial extension (Ridley et al., 1992) and Rac1-deficient macrophages have defects in CSF-1-induced membrane ruffling and spreading (Wells et al., 2004).

The Vav proteins can catalyse exchange of guanine nucleotide on several Rho proteins (reviewed in (Bustelo, 1997)). Vav proteins could be utilised to transduce signals from the CSF-1 receptor, to the Rho GTPases. However, CSF-1 also affects macrophage differentiation, survival and proliferation (Stanley et al., 1997; Rohrschneider et al., 1997) by signalling to proteins such as ERK1/2. These processes are regulated through the formation of the CSF-1R signalling complex, which is organised by signalling adapter proteins such as Grb-2 and Vav (Yeung and Stanley, 2003). Thus, Vav proteins could contribute to CSF-1R signalling in macrophages either as Rho family GEFs or as signalling adapters, in processes affecting migration or proliferation/survival.

Using macrophages derived from mice lacking single ( $Vav1^{-/-}$ ,  $Vav2^{-/-}$ ,  $Vav3^{-/-}$ ) or all three ( $Vav1/2/3^{-/-}$ ) isoforms of Vav, the role of these proteins in CSF-1-induced signal transduction, morphological responses and migration was investigated. In contrast to chapter 3, responses of macrophages to the chemo-attractant CSF-1 were compared by CSF-1 stimulation of CSF-1-deprived BMM.

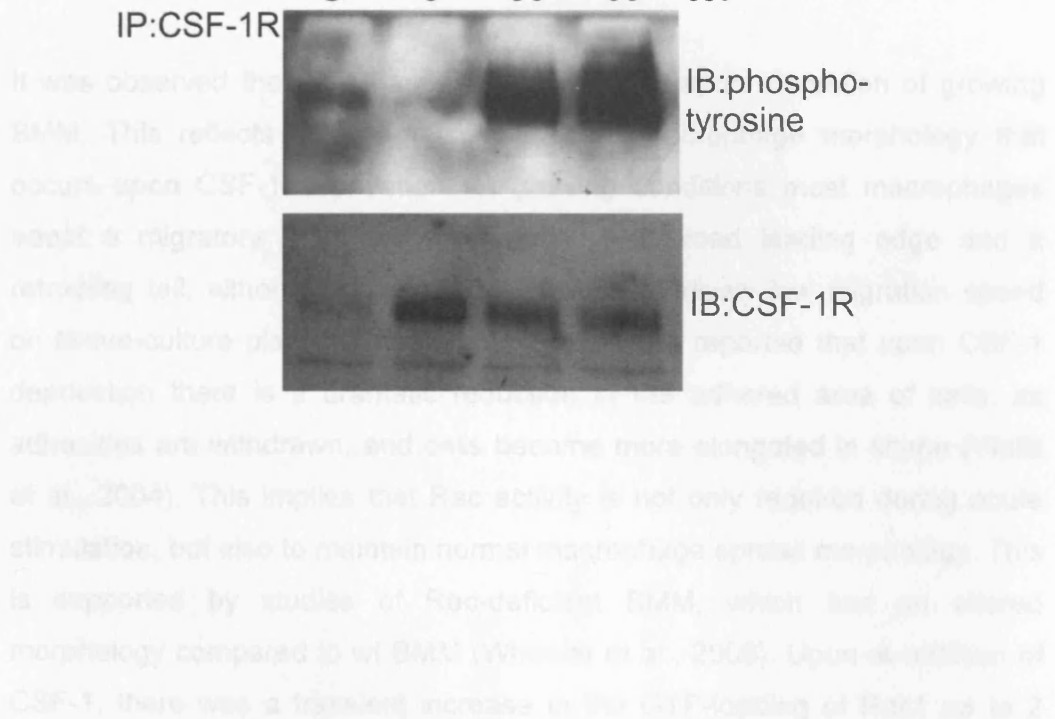


#### 4.2: CSF-1 induces CSF-1R tyrosine phosphorylation in CSF-1-starved macrophages

In order to confirm responsiveness of BMM to CSF-1, the activation of the CSF-1R upon stimulation with CSF-1 was tested at the biochemical level. The CSF-1 receptor was immunoprecipitated from BMM stimulated with CSF-1, and the activation of the receptor was analysed by its tyrosine phosphorylation levels (Figure 4.1). It was observed that, as previously reported, CSF-1 deprivation of macrophages for 16 hours led to an upregulation of the CSF-1 receptor (Li and Stanley, 1991) (lower panel). However, upon stimulation with CSF-1, the levels of the receptor appeared to decrease, as early as 30 seconds. In contrast, the tyrosine phosphorylation of the receptor was reduced upon CSF-1 deprivation. With the addition of CSF-1 to starved cells, the phosphorylation of the receptor was greatly increased by 30 seconds, and there was a further increase at 1 minute. Interestingly, the phospho-tyrosine antibody recognised a diffuse band at ~170 kDa, after stimulation, whereas it was more distinct in growing BMM. These could be the activated forms of the receptor (Li and Stanley, 1991). However, these proteins were not recognised by the CSF-1 receptor antibody and could therefore be due to co-precipitation of receptor-associated tyrosine-phosphorylated proteins. This confirmed the activation of CSF-1 signalling in macrophages upon addition of CSF-1.

## 4.3 CSF-1 activates Rac1 in macrophages

The activation of Rac1 upon stimulation with CSF-1 in BMM was determined using a Rac pull-down assay, which selectively precipitates GTP-bound Rac from cell lysates (Figure 2).



**Figure 4.1: Stimulation of BMM with CSF-1 leads to CSF-1R tyrosine phosphorylation.** Wt BMM were cultured in growth medium (G) or deprived of CSF-1 for 16 hours. Starved BMM were then stimulated with 33 ng/mL CSF-1 for the indicated times before being solubilised. Lysates were then incubated with anti-CSF-1R antibody. Immunoprecipitates were resolved by SDS-PAGE. Receptor tyrosine phosphorylation was analysed by western blotting with an anti-phosphotyrosine (clone 4G10) antibody (upper panel). Membranes were stripped and re-probed with an anti-CSF-1 receptor antibody (lower panel). Results shown are representative of three independent experiments.

### 4.3: CSF-1 activates Rac1 in macrophages

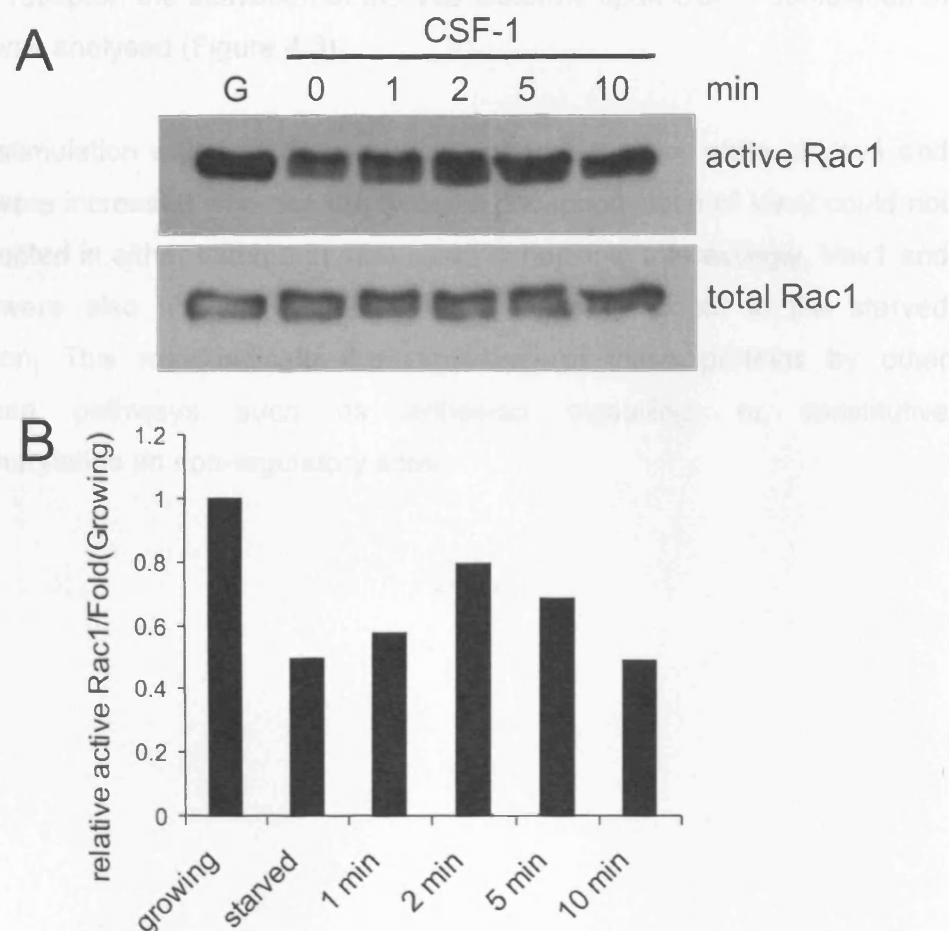
The activation of Rac1 upon stimulation with CSF-1 in BMM was determined using a Rac pull-down assay, which selectively precipitates GTP-bound Rac from cell lysates (Figure 4.2).

It was observed that Rac1 activity was reduced upon starvation of growing BMM. This reflects the dramatic change in macrophage morphology that occurs upon CSF-1 deprivation. In growing conditions most macrophages adopt a migratory morphology, comprising a broad leading edge and a retracting tail, although macrophages have a relatively low migration speed on tissue-culture plastic (Chapter 3). It has been reported that upon CSF-1 deprivation there is a dramatic reduction in the adhered area of cells, as adhesions are withdrawn, and cells become more elongated in shape (Wells et al., 2004). This implies that Rac activity is not only required during acute stimulation, but also to maintain normal macrophage spread morphology. This is supported by studies of Rac-deficient BMM, which had an altered morphology compared to wt BMM (Wheeler et al., 2006). Upon re-addition of CSF-1, there was a transient increase in the GTP-loading of Rac1 up to 2 minutes and then it decreased by 10 minutes.

Thus, Rac1 was shown to be activated in response to CSF-1 in BMM. The timecourse of the activation Rac1 coincides with the times that ruffling (1 minute) and spreading (5 minutes) responses to CSF-1 are observed in BMM (Figure 4.5).

#### 4.4. Vav1 and Vav3 are tyrosine phosphorylated upon CSF-1 stimulation of BMM

To investigate the possible role of Vav proteins as GEFs downstream of the CSF-1 receptor, the activation of the Vav isoforms upon CSF-1 stimulation of BMM was analysed (Figure 4.2).



**Figure 4.2: Stimulation of BMM with CSF-1 results in an increase in the GTP-loading of Rac1.** Wt BMM were cultured in growth medium (G) or deprived of CSF-1 for 16 hours. Starved BMM were then stimulated with 33 ng/mL CSF-1 for the indicated times before solubilisation. Lysates were incubated with glutathione sepharose beads complexed with GST-PBD fusion protein. (A) Precipitates were resolved by SDS-PAGE in parallel with WCL samples, followed by western blotting with a Rac1 antibody. (B) Rac1 activation was estimated by comparing levels of precipitated Rac1 to total Rac1 levels in the WCL samples by densitometry. Results shown are representative of two independent experiments.

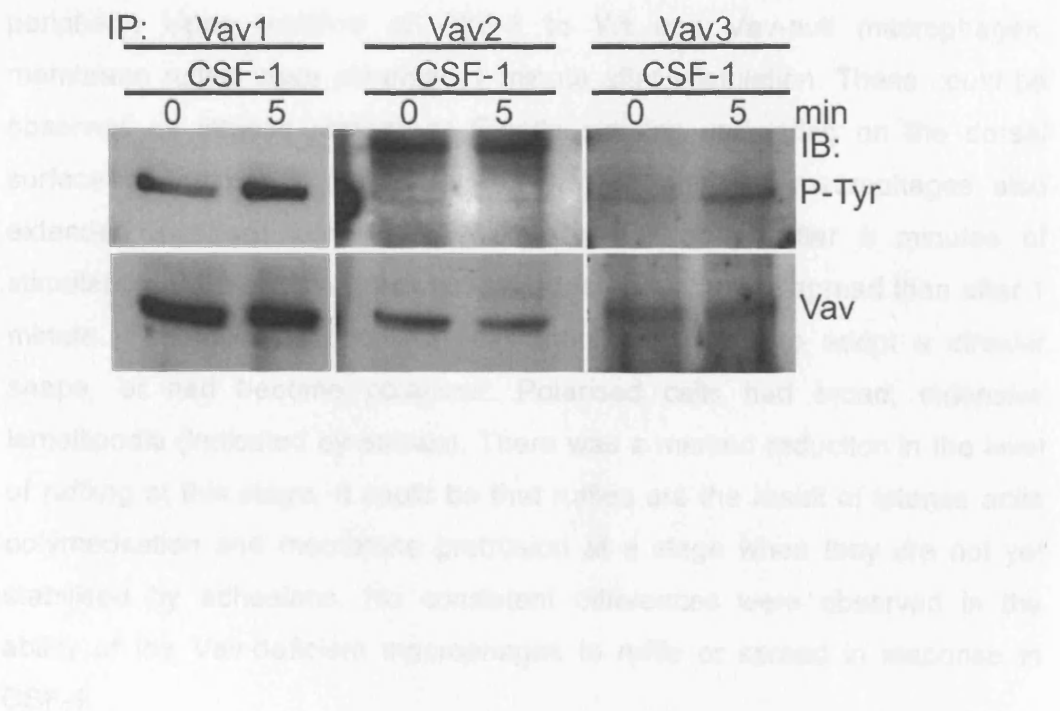
#### 4.4: Vav1 and Vav3 are tyrosine phosphorylated upon CSF-1 stimulation of BMM

To investigate the possible role of Vav proteins as GEFs downstream of the CSF-1 receptor, the activation of the Vav isoforms upon CSF-1-stimulation of BMM was analysed (Figure 4.3).

Upon stimulation with CSF-1 the tyrosine phosphorylation state of Vav1 and Vav3 were increased whereas the tyrosine phosphorylation of Vav2 could not be detected in either starved or stimulated conditions. Interestingly, Vav1 and Vav3 were also tyrosine phosphorylated to some extent in the starved condition. This may indicate the regulation of these proteins by other signalling pathways such as adhesion signalling, or constitutive phosphorylation on non-regulatory sites.

#### 4.3 Vav-deficient macrophages have normal morphological responses to CSF-1 stimulation

The predominant role of Vav proteins in CSF-1-induced rolling and spreading was then investigated using macrophages lacking extracellular domain isoforms (Figure 4.4), or all three isoforms of Vav (Figure 4.5). All the macrophage populations behaved in a similar way in response to CSF-1-dependent rolling and spreading and appeared to spread then growing cells (Figure 3.1). Any protrusions were small and limited to small regions of the cell



**Figure 4.3: Tyrosine phosphorylation of Vav1 and Vav3, but not Vav2, is increased upon stimulation of macrophages with CSF-1.** Wt BMM were cultured in growth medium and then deprived of CSF-1 for 16 hours. Starved BMM were then stimulated with 33 ng/mL CSF-1 for 5 minutes before solubilisation. Cell lysates were then incubated with antibodies specific for each of the Vav isoforms. Precipitates were resolved by SDS-PAGE, followed by western blotting with an anti-phosphotyrosine (clone 4G10) antibody. Membranes were then stripped and re-probed with antibodies specific for the respective Vav isoform. Results are representative of two independent experiments.

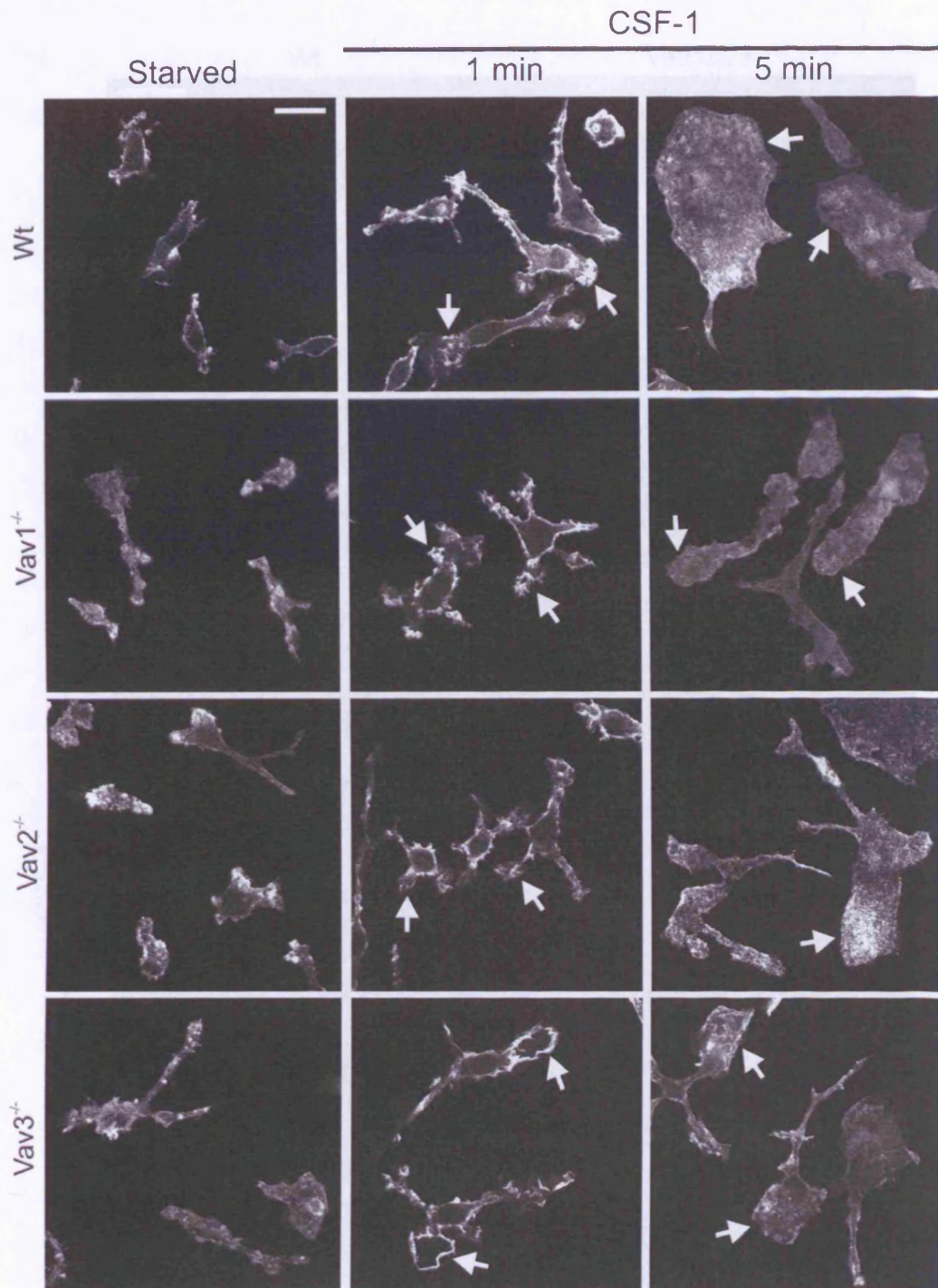
#### 4.5: Vav-deficient macrophages have normal morphological responses to CSF-1 stimulation

The requirement of Vav proteins in CSF-1-induced ruffling and spreading was then investigated using macrophages lacking expression of single isoforms (Figure 4.4), or all three isoforms of Vav (Figure 4.5). All the macrophage populations behaved in a similar way in response to CSF-1-deprivation. Cells became rounded and appeared much less spread than growing cells (Figure 3.1). Any protrusions were small and limited to small regions of the cell periphery. Upon addition of CSF-1 to Wt and Vav-null macrophages, membrane ruffles were observed 1 minute after stimulation. These could be observed as intense regions of F-actin staining undulating on the dorsal surface of the cell (indicated by arrows). In addition, macrophages also extended localised lamellipodia from the cell body. After 5 minutes of stimulation, Wt and Vav-null populations were much more spread than after 1 minute. Populations comprised cells that had spread to adopt a circular shape, or had become polarised. Polarised cells had broad, extensive lamellipodia (indicated by arrows). There was a marked reduction in the level of ruffling at this stage. It could be that ruffles are the result of intense actin polymerisation and membrane protrusion at a stage when they are not yet stabilised by adhesions. No consistent differences were observed in the ability of the Vav-deficient macrophages to ruffle or spread in response to CSF-1.

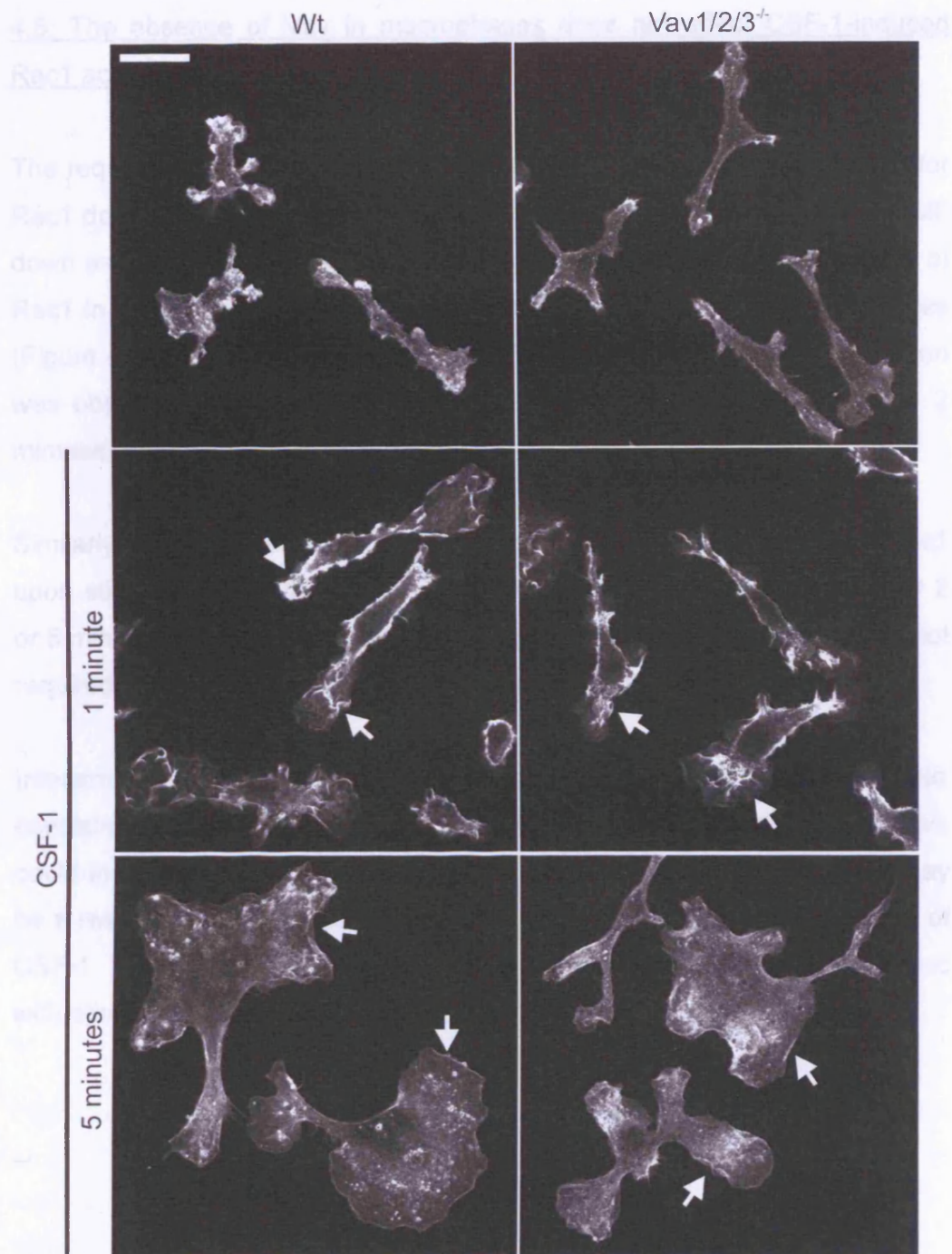
Ruffling and spreading responses to CSF-1 stimulation were then analysed in macrophages lacking all three isoforms of Vav (Figure 4.5). Both Wt and Vav1/2/3<sup>-/-</sup> BMM behaved similarly to CSF-1 deprivation. By one minute of CSF-1 stimulation, F-actin ruffles were observed on the dorsal surfaces of the Wt and Vav1/2/3<sup>-/-</sup> macrophages (indicated by arrows). As well as ruffles cell protrusions in the form of lamellipodia were also observed at one minute. After 5 minutes of treatment with CSF-1, a clear increase in the size of cell protrusions was observed in the Vav1/2/3<sup>-/-</sup> macrophages (indicated by arrows). Thus, in the absence of all three isoforms of Vav proteins

macrophages retain the ability to ruffle and spread in response to CSF-1 stimulation.





**Figure 4.4: BMM ruffling and spreading in responses to CSF-1 stimulation are unaffected by the absence of single Vav isoforms.** Wt, Vav1<sup>-/-</sup>, Vav2<sup>-/-</sup> and Vav3<sup>-/-</sup> BMM were cultured on glass coverslips for 24 hours, then starved of CSF-1 for 16 hours. Starved cells were then stimulated with 33ng/mL CSF-1 for 1 or 5 minutes before cells were fixed, and stained for F-actin with TRITC-phalloidin. Images were acquired by laser scanning confocal microscopy. Bar=20  $\mu$ m. Arrows indicate membrane ruffles (middle column) and lamellipodia (right column). Images are representative of three independent experiments.



**Figure 4.5: BMM ruffling and spreading in response to CSF-1 stimulation are unaffected by the absence of all three Vav isoforms.** Wt and Vav1/2/3<sup>-</sup> BMM were cultured on glass coverslips for 24 hours, then starved of CSF-1 for 16 hours. Starved cells were then stimulated with 33 ng/mL CSF-1 for 1 or 5 minutes before cells were fixed, and stained for F-actin with TRITC-phalloidin. Images were acquired by laser scanning confocal microscopy. Bar=20  $\mu$ m. Arrows indicate membrane ruffles (middle row) and lamellipodia (bottom row). Images are representative of three independent experiments.

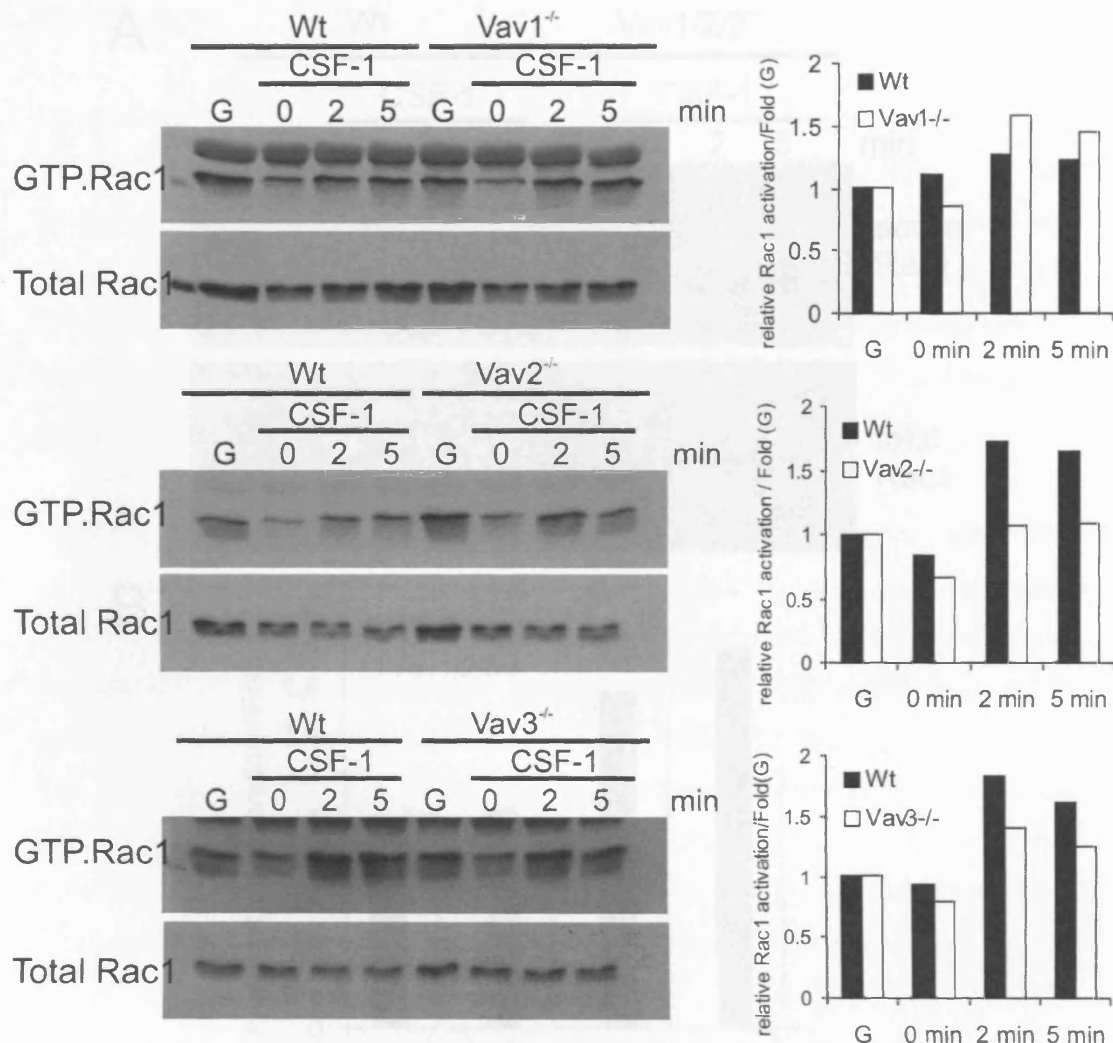
#### 4.6: The absence of Vav in macrophages does not affect CSF-1-induced Rac1 activation

The requirement of Vav proteins as guanine nucleotide exchange factors for Rac1 downstream of the CSF-1 receptor was investigated using a Rac1 pull-down assay. In macrophages lacking single isoforms of Vav, the activation of Rac1 in response to CSF-1-stimulation was comparable to Wt macrophages (Figure 4.6). In all populations the reduction of Rac1 activity upon starvation was observed. Upon addition of CSF-1, Rac1 activity was increased by 2 minutes, and maintained up to 5 minutes.

Similarly, in the absence of all three Vav isoforms, the activation of Rac1 upon stimulation with CSF-1 was not different from Wt macrophages after 2 or 5 minutes of stimulation with CSF1 (Figure 4.7). Thus, Vav proteins are not required for CSF-1-induced Rac1 activation in macrophages.

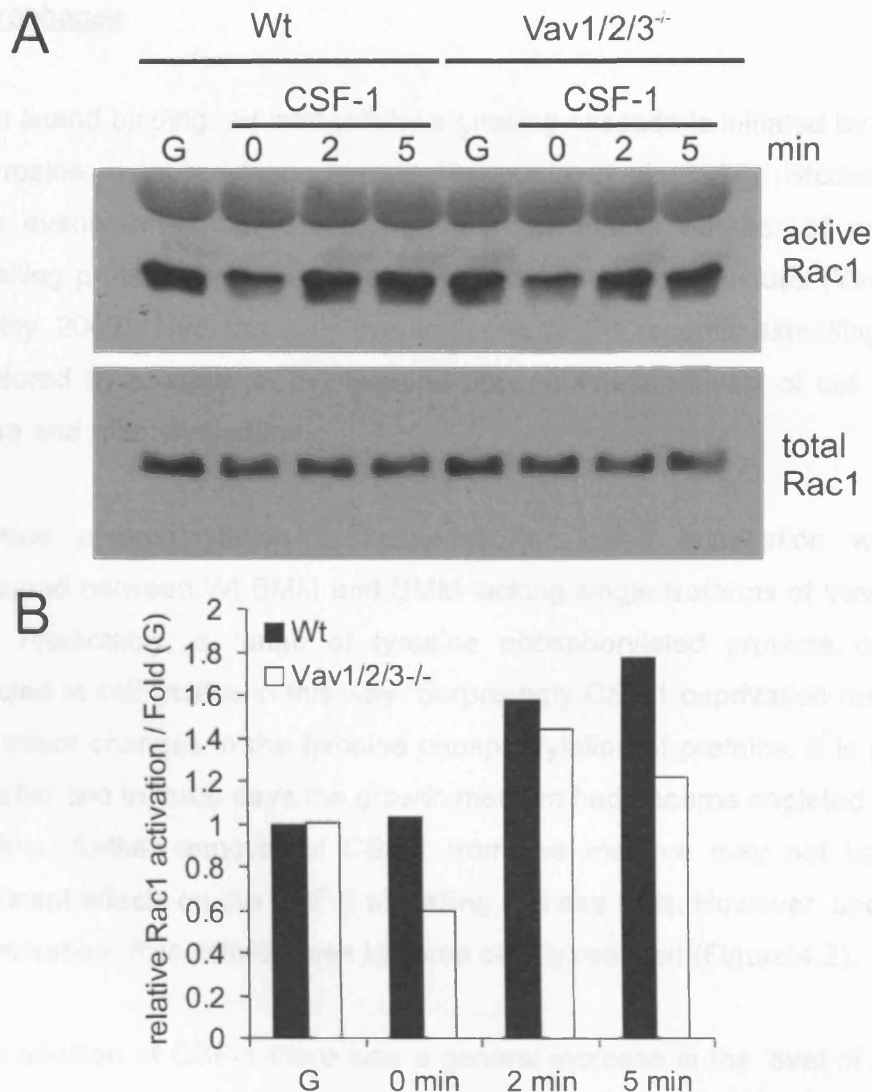
Interestingly, the levels of Rac1 expressed in CSF-1-deprived cells consistently appeared reduced in comparison to cells in growth medium. This could indicate a down-regulation of Rac upon starvation. Alternatively, it may be a result of the loss of some cells that become detached in the absence of CSF-1. This could be investigated further by the comparing the levels of Rac with other proteins, such as ERK1/2 or  $\beta$ -actin.





**Figure 4.6: CSF-1 induced activation of Rac1 is unaffected by the absence of single Vav isoforms.** Wt, Vav1<sup>-/-</sup>, Vav2<sup>-/-</sup>, and Vav3<sup>-/-</sup> BMM were cultured in growth medium (G) or deprived of CSF-1 for 16 hours. Starved BMM were then stimulated with 33 ng/mL CSF-1 for the indicated times before solubilisation. Lysates were incubated with glutathione sepharose beads complexed with GST-PBD fusion protein. Precipitates were then resolved by SDS-PAGE in parallel with WCL samples, followed by western blotting with an anti-Rac1 antibody (Left panels). Rac1 activation at different time-points was estimated by comparing levels of precipitated Rac1 to total Rac1 levels in the WCL samples by densitometry and determining the fold activation relative to (G) (Right panels). Results are representative of three independent experiments.

## 4.7. Absence of Vav proteins does not affect Vav1/2/3 response to CSF-1 in macrophages



**Figure 4.7: CSF-1-induced activation of Rac1 in macrophages is not affected by the absence of all three Vav isoforms.** Wt and *vav1/2/3*<sup>-/-</sup> BMM were cultured in growth medium (G) or deprived of CSF-1 for 16 hours. Starved BMM were then stimulated with 33 ng/mL CSF-1 for the indicated times before solubilisation. Lysates were incubated with glutathione sepharose beads complexed with GST-PBD fusion protein. (A) Precipitates were then resolved by SDS-PAGE in parallel with WCL samples, followed by western blotting with an anti-Rac1 antibody. (B) Rac1 activation at different time-points was estimated by comparing levels of precipitated Rac1 to total Rac1 levels in the WCL samples by densitometry and determining the fold activation relative to (G). Results are representative of three independent experiments.

#### 4.7: Absence of Vav proteins does not affect signalling responses to CSF-1 in macrophages

Upon ligand binding, an intracellular signalling cascade is initiated by a series of tyrosine phosphorylation events (Sengupta et al., 1998). Studies of the early events after CSF-1R activation show that a number of prominent signalling proteins become phosphorylated on tyrosine residues (Yeung and Stanley, 2003). Thus the early events during CSF-1 receptor signalling can be monitored by analysis of the tyrosine phosphorylation levels of cell proteins before and after stimulation.

Tyrosine phosphorylation in response to CSF-1 stimulation was first compared between Wt BMM and BMM lacking single isoforms of Vav (Figure 4.8). Predictably, a range of tyrosine phosphorylated proteins could be detected in cell lysates in this way. Surprisingly CSF-1 deprivation resulted in only minor changes in the tyrosine phosphorylation of proteins. It is possible that after two to three days the growth medium had become depleted of CSF-1. Thus, further removal of CSF-1 from the medium may not have had significant effects on the CSF-1 signalling in these cells. However, upon CSF-1-deprivation, Rac activity does become clearly reduced (Figure 4.2).

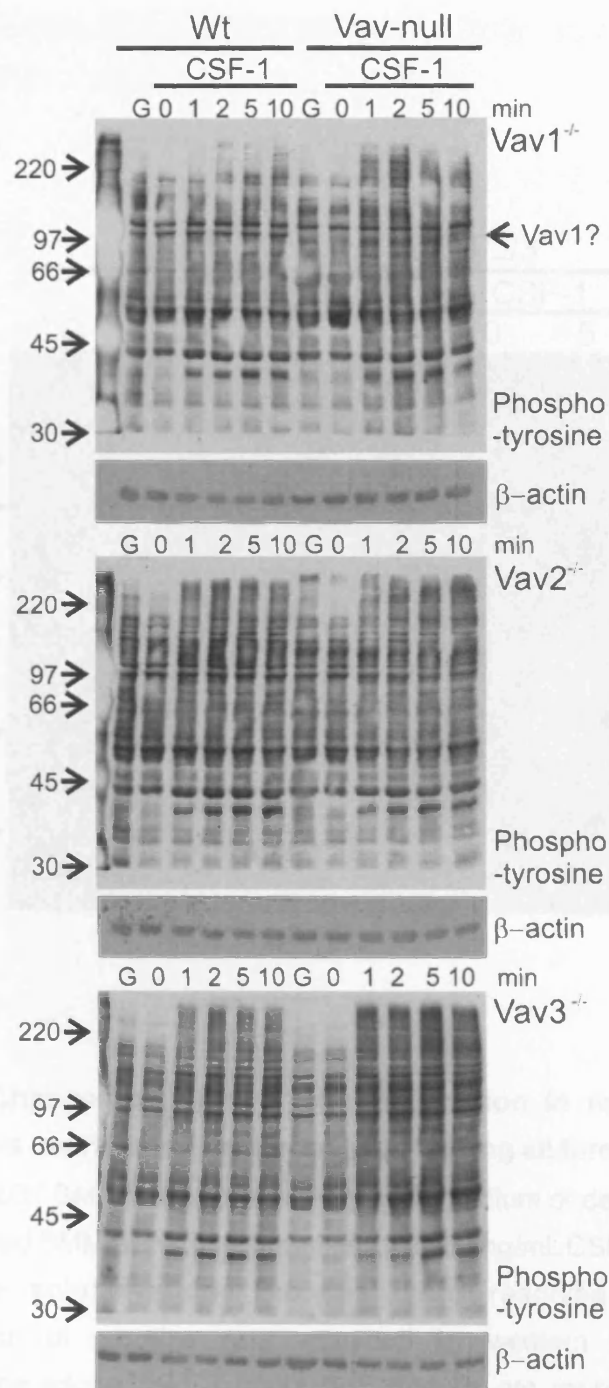
Upon addition of CSF-1 there was a general increase in the level of tyrosine phosphorylation, even as early as 1 and 2 minutes, before the cells had become spread (see Figure 4.4). This indicated the initiation of tyrosine phosphorylation events and signal transduction within cells. However, the major indicator of CSF-1R activation was the appearance of tyrosine-phosphorylated proteins at apparent molecular weights above 150 kDa. The identity of these proteins is unknown, but is likely to include activated forms of the CSF-1 receptor (Li and Stanley, 1991), which has a monomeric apparent molecular weight of approximately 165 kDa (Rettenmier et al., 1988).

This pattern of tyrosine phosphorylation changes was also observed in macrophages lacking single isoforms of Vav. No consistent differences were

observed in the extent or the timecourse of these events in the Vav-null macrophages.

Interestingly, in Wt macrophages there was a tyrosine phosphorylated protein at an apparent molecular weight of 97kDa, which is the apparent molecular weight of mammalian Vav proteins. This protein band was absent in macrophages that did not express Vav1, but was present in Vav2<sup>-/-</sup> and Vav3<sup>-/-</sup> macrophages (Figure 4.8 upper panel). This band could therefore be Vav1, or a protein that is dependent on Vav1 to maintain its tyrosine phosphorylation state or expression.

In macrophages lacking all three Vav proteins, the pattern of tyrosine phosphorylation in response to CSF-1 stimulation was indistinguishable from Wt BMM (Figure 4.9). In general, there was an increase in the phosphorylation of proteins upon stimulation. Furthermore, the extent of this increase was similar in Vav1/2/3<sup>-/-</sup> and Wt macrophages. Tyrosine phosphorylation of proteins in both populations was more evident in proteins above apparent molecular weights of 100 kDa, and at 40-42 kDa. Incidentally in Vav1/2/3<sup>-/-</sup> macrophages the tyrosine-phosphorylated protein at 95 kDa, which is present in Wt macrophages is absent. Again, this could be due to the absence of Vav1, as in Vav1<sup>-/-</sup> macrophages.



**Figure 4.8: Changes in tyrosine phosphorylation in response to CSF-1 stimulation are unaffected in macrophages lacking single isoforms of Vav.** Wt, Vav1<sup>-/-</sup>, Vav2<sup>-/-</sup> and Vav3<sup>-/-</sup> BMM were cultured in growth medium (G) or deprived of CSF-1 for 16 hours. Starved BMM were then stimulated with 33 ng/mL CSF-1 for the indicated times before solubilisation. Proteins were resolved by SDS-PAGE. Tyrosine phosphorylation of proteins was analysed by western blotting with an anti-phosphotyrosine (clone 4G10) antibody. Total protein loading was analysed by re-probing blots with an anti-β-actin antibody. Results are representative of three independent experiments.



### 4.8 CSF-1-induced ERK1/2 activation is not affected by the absence of Vav proteins in BMDM-12 cells

The ERK1/2 signalling cascade is involved in regulating a variety of cellular

processes (see review by Blumberg and Blumberg, 1999). The activation of

which are regulated by a variety of growth factors and cytokines through

receptor tyrosine kinases (RTKs) and other signalling molecules.

activation of

Vav1/2/3

(Fukui et al., 1994) is involved in the activation of ERK1/2 in response to CSF-1

receptor

CSF-1

phosphorylation

in cells

at 2 and 5 min

activation is

macrophages that lack all three isoforms of Vav. The activation of ERK1/2 in

Vav<sup>1/2/3</sup> macrophages was also comparable in both extent and timing

to that observed in Vav macrophages (Figure 4.10). This result shows that

the activation of ERK1/2 is not dependent on Vav proteins in BMDM-12 cells.

Figure 4.9: Changes in tyrosine phosphorylation in response to CSF-1

stimulation are unaffected in macrophages lacking all three isoforms of Vav.

Wt and Vav1/2/3<sup>+</sup> BMM were cultured in growth medium or deprived of CSF-1 for

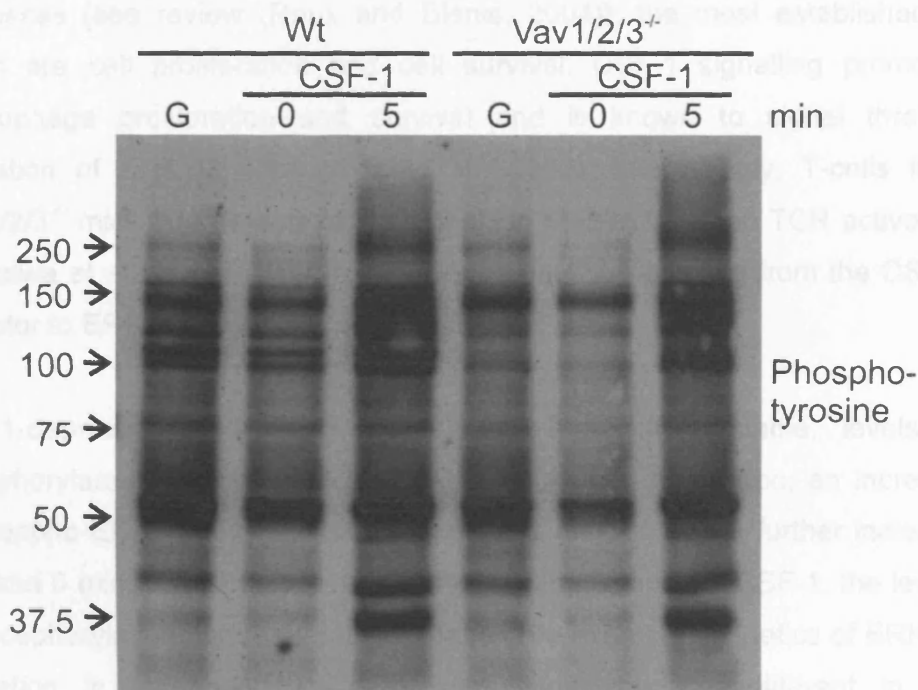
16 hours. Staved BMM were then stimulated with 33 ng/mL CSF-1 for the indicated

times before solubilisation. Proteins were resolved by SDS-PAGE.

Phosphorylation of proteins was analysed by western blotting with anti-

phosphotyrosine (clone 4G10) antibody. Results are representative of three

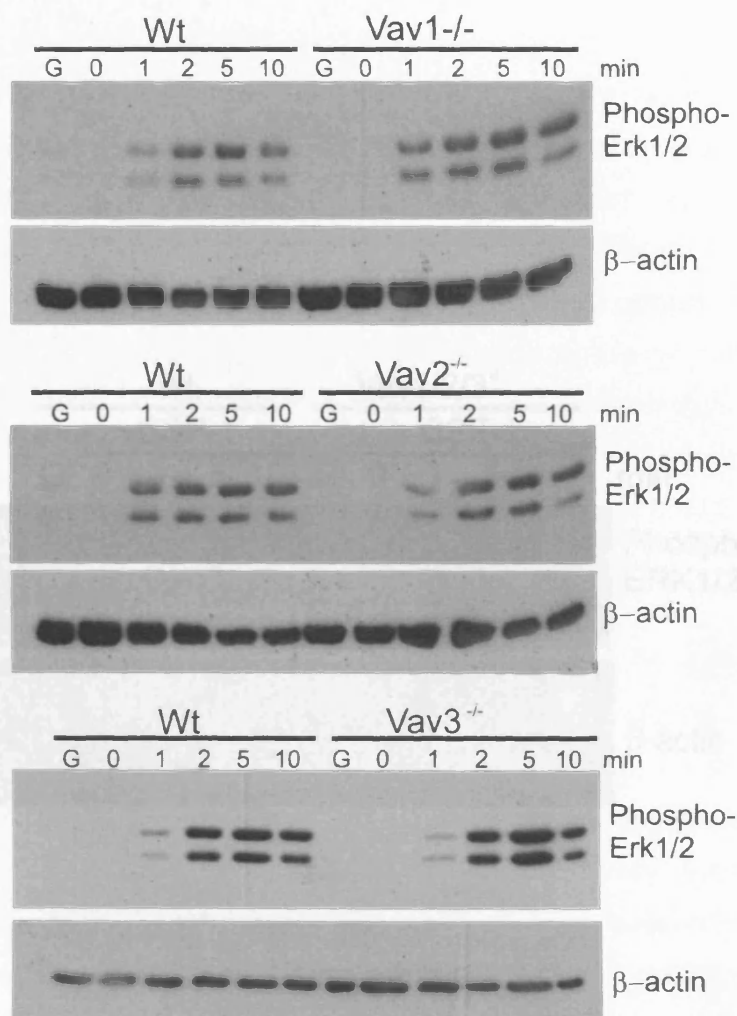
independent experiments.



#### 4.8: CSF-1-induced ERK1/2 activation is not affected by the absence of Vav proteins in macrophages

The ERK1/2 signalling cascade is involved in regulating a variety of cellular processes (see review (Roux and Blenis, 2004)), the most established of which are cell proliferation and cell survival. CSF-1 signalling promotes macrophage proliferation and survival and is known to signal through activation of ERK1/2 (Comalada et al., 2003). Interestingly, T-cells from *Vav1/2/3<sup>-/-</sup>* mice had defects in the activation of ERK1/2 upon TCR activation (Fujikawa et al., 2003). Vav protein requirement in signalling from the CSF-1 receptor to ERK1/2 was therefore investigated.

CSF-1-deprived macrophages had low, almost undetectable, levels of phosphorylated ERK1/2 (Figure 4.10). Upon CSF-1-stimulation, an increase in phospho-ERK1/2 levels was observed at 1 minute, with a further increase at 2 and 5 minutes. But after 10 minutes of stimulation with CSF-1, the levels of phosphorylation had begun to decrease. The extent and kinetics of ERK1/2 activation in response to CSF-1 stimulation was not different in the macrophages that lacked single isoforms of Vav. The activation of ERK1/2 in *Vav1/2/3<sup>-/-</sup>* macrophages was also comparable in both extent and timecourse to that observed in Wt macrophages (Figure 4.11). Thus it was shown that Vav proteins are not required for CSF-1 signalling to the ERK1/2 pathway.



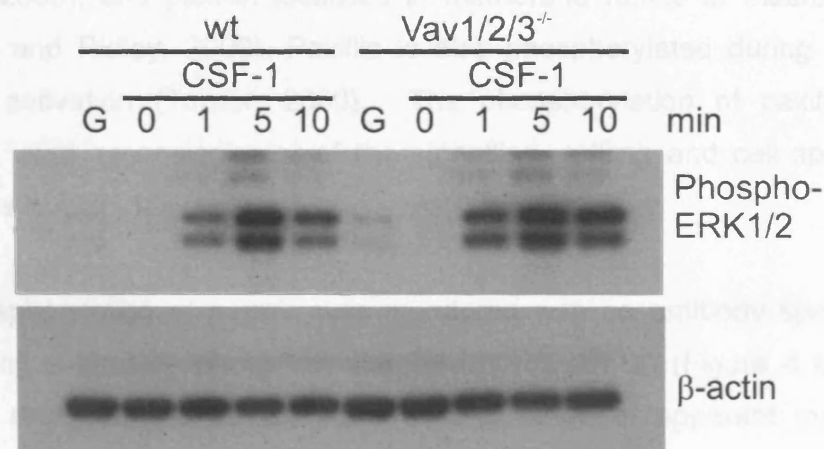
**Figure 4.10: ERK1/2 phosphorylation upon CSF-1 stimulation of macrophages is not affected by the absence of single isoforms of Vav.** Wt, Vav1<sup>-/-</sup>, Vav2<sup>-/-</sup> and Vav3<sup>-/-</sup> BMM were cultured in growth medium (G) or deprived of CSF-1 for 16 hours. Starved BMM were then stimulated with 33 ng/mL CSF-1 for the indicated times before solubilisation. Proteins were resolved by SDS-PAGE. Activation of ERK1/2 was analysed by western blotting with a polyclonal anti-phospho-ERK1/2 (Tyr202, Thr204) antibody. Total protein loading was analysed by re-probing blots with an anti-β-actin antibody. Results shown are representative of three independent experiments.

#### 4.9 Vav proteins are not required for CSF-1 induced phosphorylation of ERK1/2

The requirement of Vav proteins as signalling adaptors in the CSF-1-induced signalling complex was investigated further by analysis of changes in the phosphorylation of ERK1/2. Paxillin has been identified as one of the components of the CSF-1 signalling complex in macrophages. Activation of the CSF-1 receptor results in tyrosine phosphorylation of paxillin (Young and Stanley, 2003), and paxillin localises in membrane ruffles of macrophages (Mullins and Burstein, 2003). Paxillin is recruited to the CSF-1 receptor during integrin-mediated growth factor signalling.

Macrophages were grown in the presence of CSF-1 for 16 hours and then starved for 16 hours. Starved macrophages were then stimulated with CSF-1 for the indicated times before solubilisation. Proteins were resolved by SDS-PAGE. Activation of ERK1/2 was analysed by western blotting with a polyclonal anti-phospho-ERK1/2 (Tyr202, Thr204) antibody.

The phosphorylation of ERK1/2 was analysed by western blotting with a polyclonal anti-phospho-ERK1/2 (Tyr202, Thr204) antibody. Total protein loading was analysed by re-probing blots with an anti- $\beta$ -actin antibody. Results shown are representative of two independent experiments.



**Figure 4.11: ERK1/2 phosphorylation upon CSF-1 stimulation of macrophages is not affected by the absence of all three isoforms of Vav.** Wt and Vav1/2/3<sup>-/-</sup> BMM were cultured in growth medium or deprived of CSF-1 for 16 hours. Starved BMM were then stimulated with 33 ng/mL CSF-1 for the indicated times before solubilisation. Proteins were resolved by SDS-PAGE. Activation of ERK1/2 was analysed by western blotting with a polyclonal anti-phospho-ERK1/2 (Tyr202, Thr204) antibody. Total protein loading was analysed by re-probing blots with an anti- $\beta$ -actin antibody. Results shown are representative of two independent experiments.

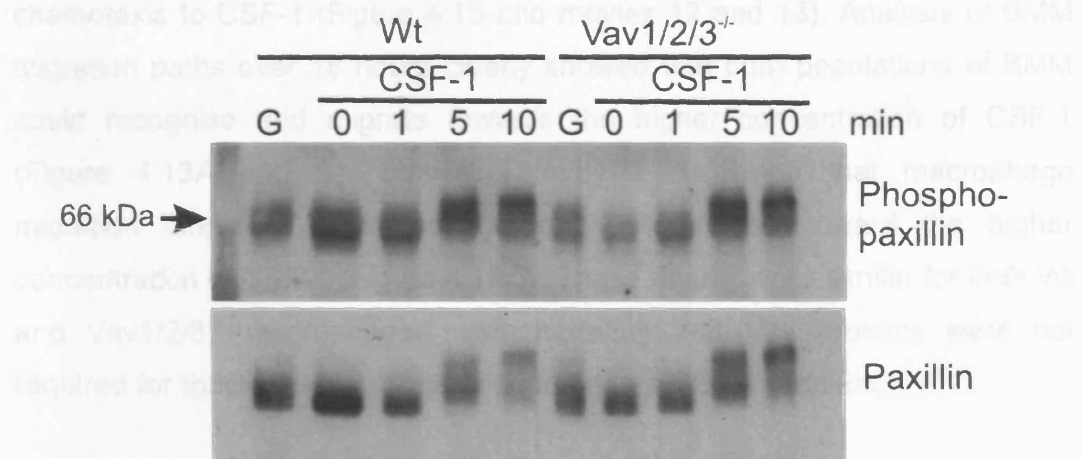
#### 4.9: Vav proteins are not required for CSF-1 induced phosphorylation of paxillin

The requirement of Vav proteins as signalling adaptors in the CSF-1 signalling complex was investigated further by analysis of changes in the phosphorylation of paxillin. Paxillin has been identified as one of the components of the CSF-1 signalling complex in macrophages. Activation of the CSF-1 receptor results in tyrosine-phosphorylation of paxillin (Yeung and Stanley, 2003), and paxillin localises in membrane ruffles of macrophages (Williams and Ridley, 2000). Paxillin is also phosphorylated during integrin receptor activation (Turner, 2000). The phosphorylation of paxillin was therefore used as an indicator of the signalling, ruffling and cell spreading responses to CSF-1.

The phosphorylation of paxillin was monitored with an antibody specifically recognising a tyrosine phosphorylated site (tyrosine118) (Figure 4.12). The antibody recognised a diffuse band over a range of apparent molecular weights, from 65 kDa to approximately 70 kDa. This range of mobilities of paxillin is due to phosphorylation of different residues (Turner, 2000; Bellis et al., 1997). In both Wt and Vav1/2/3<sup>-/-</sup> macrophages cultured in growth medium, paxillin existed uniformly across its different mobilities. Upon starvation it became concentrated in a higher mobility state. At 1 minute of stimulation with CSF-1 there was no observable change in its phosphorylation state. But by 5 minutes much of tyrosine118-phosphorylated paxillin was in a lower mobility form. By 10 minutes tyrosine118-phosphorylated paxillin was concentrated in a distinct band at the lowest mobility. This pattern of changes in response to CSF-1 stimulation was consistent for both Wt and Vav1/2/3<sup>-/-</sup> macrophages. Thus, paxillin phosphorylation was not different in Vav1/2/3<sup>-/-</sup> macrophages in response to CSF-1 stimulation.

#### 4.10 Vav proteins are not required for macrophage chemotaxis to CSF-1

Results so far indicate that Vav proteins are not required in early morphological and signalling responses of macrophages to CSF-1 stimulation. Next, the role of Vav proteins in CSF-1-induced macrophage chemotaxis was investigated. To this end, a Chemotaxis chamber assay (Wells et al., 1996) was utilised to compare Wt and Vav1/2/3<sup>-/-</sup> macrophage chemotaxis to CSF-1 (Figure 4.13 and Table 4.13). Analysis of BMM migration paths over 16 h revealed that both populations of BMM could respond to CSF-1 (Figure 4.13A).



**Figure 4.13:** Vav1/2/3<sup>-/-</sup> BMM have similar chemotactic responses to CSF-1 as Wt BMM. Wt and Vav1/2/3<sup>-/-</sup> BMM were cultured in growth medium or deprived of CSF-1 for 16 hours. Staved BMM were then stimulated with 33 ng/mL CSF-1 for the indicated times before solubilisation. Proteins were resolved by SDS-PAGE. Phosphorylation of paxillin was analysed by western blotting with an anti-Y118-phospho-paxillin antibody. Total protein loading was analysed by re-probing blots with an anti-paxillin antibody. Results are representative of two independent experiments.

**Figure 4.12: Changes in the tyrosine phosphorylation of paxillin in response to CSF-1 stimulation are unaffected in macrophages lacking all three isoforms of Vav.** Wt and Vav1/2/3<sup>-/-</sup> BMM were cultured in growth medium or deprived of CSF-1 for 16 hours. Staved BMM were then stimulated with 33 ng/mL CSF-1 for the indicated times before solubilisation. Proteins were resolved by SDS-PAGE. Phosphorylation of paxillin was analysed by western blotting with an anti-Y118-phospho-paxillin antibody. Total protein loading was analysed by re-probing blots with an anti-paxillin antibody. Results are representative of two independent experiments.

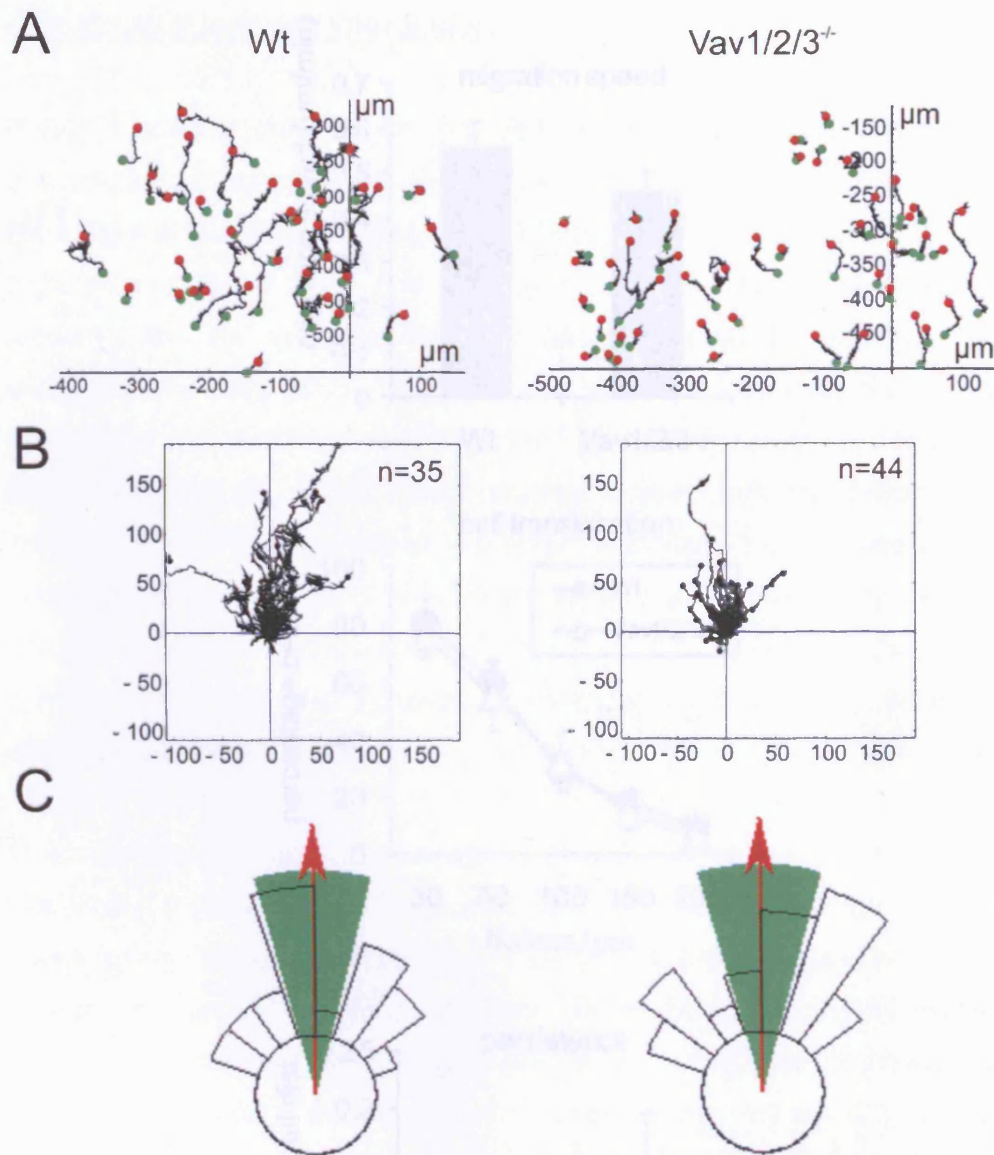
#### 4.10: Vav proteins are not required for macrophage chemotaxis to CSF-1

Results so far indicate that Vav proteins are not required in early morphological and signalling responses of macrophages to CSF-1 stimulation. Next the role of Vav proteins in CSF-1-induced macrophage chemotaxis was investigated. To this end, a Dunn chemotaxis chamber assay (Webb et al., 1996) was utilised to compare Wt and Vav1/2/3<sup>-/-</sup> macrophage chemotaxis to CSF-1 (Figure 4.13 and movies 12 and 13). Analysis of BMM migration paths over 18 hours clearly showed that both populations of BMM could recognise and migrate towards the higher concentration of CSF-1 (Figure 4.13A and B). Statistical analysis confirmed that macrophage migration directions were not random but directed toward the higher concentration of CSF-1 (Figure 4.13C). These results were similar for both Wt and Vav1/2/3<sup>-/-</sup> macrophages, demonstrating that Vav proteins were not required for macrophage directed migration in a CSF-1 gradient.

#### 4.11: Vav1/2/3<sup>-/-</sup> BMM have slightly reduced translocation, but enhanced migration persistence during chemotaxis

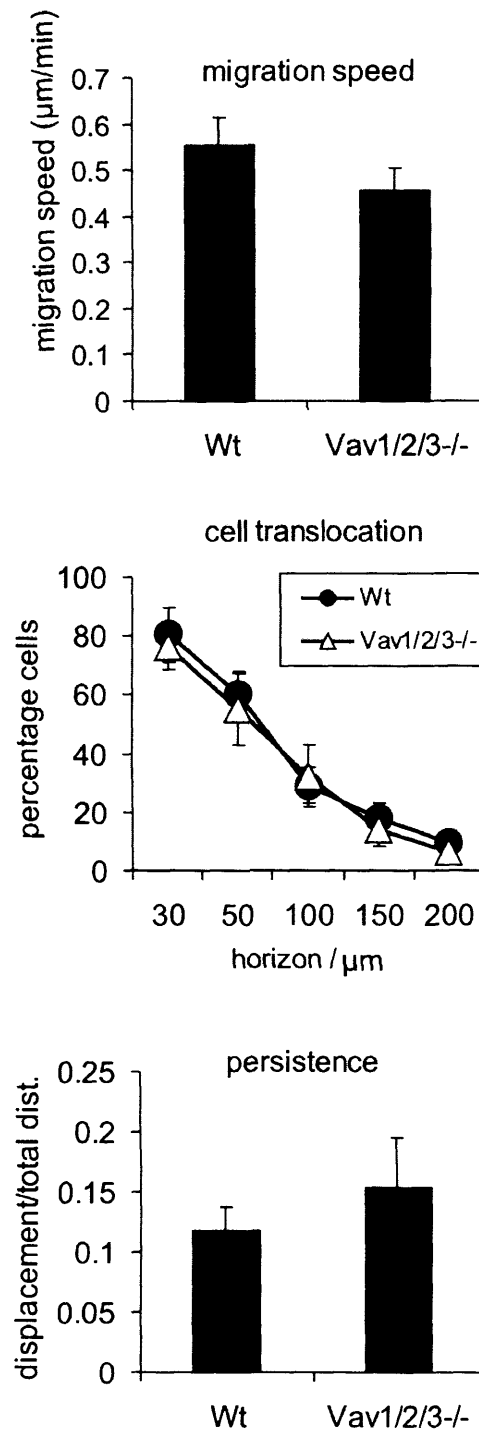
Analysis of macrophage migration and morphology of Vav1/2/3<sup>-/-</sup> macrophages showed that these cells had an altered morphology that affected their migration properties (Chapter 3). In order to study if this affected their migration in a gradient of chemo-attractant, the data from the chemotaxis study was further analysed for migration speed, cell translocation and migration persistence during chemotaxis.

Though the differences were not statistically significant, Vav1/2/3<sup>-/-</sup> macrophage populations had reduced migration speed, and enhanced migration persistence over four experiments. Cell translocation did not appear to be affected. This correlated with the migration properties of Vav1/2/3<sup>-/-</sup> macrophages in the absence of a chemo-attractant gradient (Chapter 3).



**Figure 4.13: Macrophage chemotaxis to CSF-1 is unaffected by the absence of all three isoforms of Vav.** Wt and Vav1/2/3<sup>-/-</sup> BMM were cultured on glass coverslips for 24 hours before being deprived of CSF-1 for 6 hours. Coverslips were then inverted over the bridge region of the Dunn chamber slide. A CSF-1 gradient was established by addition of 33 ng/mL CSF-1 to the outer well. Macrophages exposed to the gradient were then filmed by time-lapse phase contrast microscopy, acquiring images every 5 minutes for 18 hours (movies 12 and 13). (A) and (B) Migration of individual cells was tracked by Kinetic Imaging tracking software, and analysed using Mathematica software. (C) Rayleigh test for significant clustering of migration directions for cells migrating 30  $\mu\text{m}$  or more was carried out to determine significant chemotaxis of the cell population (green shaded segment indicates 95% confidence level). Results are representative of four independent experiments.





**Figure 4.14: Vav1/2/3<sup>-/-</sup> BMM have slightly reduced migration speed, and increased migration persistence during chemotaxis to CSF-1.** Migration data generated during the chemotaxis experiments was analysed for (A) migration speed, (B) cell translocation: numbers of cells translocating a certain distance (horizon) were quantified, and (C) migration persistence: the ratio of cell displacement to total distance migrated for each population. Data were pooled from four independent experiments (n>20 for each population).

**4.12: Conclusions and Discussion**

Previous studies have shown that Vav proteins are important in signalling downstream of activated cell surface receptors. Vav1 is required for murine bone marrow cell chemotaxis to SDF-1 (Whetton et al., 2003), and neutrophil responses to fMLP (Kim et al., 2003). Vav1 and Vav3 function was also reported to be required for complement-mediated phagocytosis in macrophages (Hall et al. 2006). A number of independent studies have indicated a role for Vav2 downstream of the EGF receptor (Marcoux et al., 2003; Tamas et al., 2003). These studies suggest that Vav proteins are a means of linking cell-surface receptor activation to regulation of the cytoskeleton through the Rho GTPase family. Colony-stimulating factor is a macrophage chemo-attractant and growth factor. In light of the previous research, the role Vav proteins downstream of the CSF-1 receptor in macrophages was investigated.

The activation of the CSF-1 receptor upon CSF-1 stimulation was verified. The levels of the receptor increase upon CSF-1 deprivation and interestingly appear to decrease rapidly upon stimulation. Cbl ubiquitin ligase is one of the proteins known to associate with the CSF-1 receptor upon its activation (Yeung and Stanley, 2003). Protein ubiquitination can lead to protein down-regulation by internalisation and also degradation (review (Dikic, 2003)). However the decrease observed is unlikely to be due to degradation of the protein since the time between the conditions is so short. The apparent reduction in the levels of the receptor may be due to receptor internalisation to a cellular compartment not solubilised during the lysis procedure. Indeed internalisation of the CSF-1 receptor within minutes of stimulation has been reported in Bacl.2F5 macrophage cell line (Li et al., 1991) and study of Cbl-null BMM showed that CSF-1 receptor internalisation upon stimulation was delayed (Lee et al., 1999). Alternatively, poor stripping of the phosphotyrosine antibody may have resulted in reduced binding of the anti-CSF1 receptor antibody. Probing for the receptor, before probing for phosphotyrosine would rectify this.

The role of Vav proteins in macrophage morphological responses to CSF-1 stimulation was investigated. The results showed that when single isoforms or all isoforms of Vav are not expressed, macrophages activate Rac1 normally, and make the appropriate morphological changes, upon CSF-1 stimulation. This suggests that Vav proteins are not required to catalyse guanine nucleotide exchange on Rac1 during CSF-1 induced cytoskeletal changes.

In macrophages not expressing Vav1, there is a distinct absence of a 97 kDa tyrosine-phosphorylated protein, as shown by western blots of cellular tyrosine phosphorylated proteins. It is likely that this protein is Vav1. In Wt, Vav2<sup>-/-</sup>, and Vav3<sup>-/-</sup> macrophages the tyrosine phosphorylation state at this apparent molecular weight was unchanged in growing, starved or CSF-1-stimulated conditions. Thus, by analysis of whole cell lysates, it appeared that Vav1 was not regulated downstream of the CSF-1 receptor. And that Vav2 and Vav3 do not wholly compensate for the loss of Vav1 in macrophages. However, immuno-precipitation of Vav1, Vav2 and Vav3 from CSF-1 starved or stimulated macrophages, showed that the tyrosine phosphorylation of Vav1 and Vav3 is increased upon stimulation. It is possible that concentrating the proteins through precipitation allowed detection of variations in the tyrosine phosphorylation levels that was not possible in the WCL. Other studies also indicate that Vav proteins are phosphorylated by CSF-1 stimulation in macrophages. An early study of tyrosine phosphorylated proteins in macrophages in response to CSF-1 stimulation reported that an approximately 99 kDa protein was tyrosine phosphorylated upon stimulation. This study investigated the kinetics of early phosphorylation events during CSF-1 receptor activation. Experiments were carried out at 4°C and even in these conditions the phosphorylation of the protein (likely to be Vav) was very transient (Sengupta et al., 1998). In platelets, Vav itself is constitutively associated with the tyrosine phosphatase SHP-1 (SH2 domain-containing tyrosine phosphatase-1) (Jones et al., 2004). For these reasons it may be difficult to see changes in the phosphorylation state of endogenous Vav. And recent studies in osteoclasts show that both Vav1 and Vav3 could as Rac1 exchange factors upon stimulation with CSF-1 and demonstrated a stimulation-dependent association of Vav1 and Rac1 (Sakai et al., 2006).

Thus Vav1 and Vav3 could be activated by CSF-1 stimulation, although it appears that they are not acting as Rac1 GEFs. Other Rho family GTPases could also be activated downstream of the CSF-1 receptor and Vav proteins could act as GEFs for GTPases such as RhoA, Cdc42, RhoG or Rac2, in other CSF-1 induced cellular responses that have not been studied as part of this investigation. Therefore it would be interesting to investigate if these other GTPases are activated upon CSF-1 stimulation.

Though Vav1 and Vav3 were phosphorylated in response to CSF-1 stimulation, that they were also catalytically activated was not confirmed. Tyrosine phosphorylation of residues in the Ac domain of Vav regulate its GEF catalytic activity (Ch. 1.4), but only phosphorylation of certain residues have this regulatory function (Lopez-Lago et al., 2000). The antibody used to detect tyrosine phosphorylation is a general antibody recognizing phosphotyrosine sites within a common consensus sequence. It is possible that the increase in phosphorylation was on tyrosine sites that are not regulatory. Antibodies specifically recognizing the regulatory tyrosine residues could be used to resolve this issue.

Another consideration is the signaling stimuli that the macrophages are exposed to during the response to CSF-1. Care was taken to ensure that responses to CSF-1 could not be attributed to other factors, for example to the re-addition of fresh serum to CSF-1-starved macrophages. However, it is possible that the Vav1 and Vav3 phosphorylation could, in part, be due to signals from the formation of new adhesions, as the cells spread in response to CSF-1. This possible contribution to the regulation of Vav proteins was unavoidable using adherent macrophages but would not be an issue when stimulating cells in suspension (Vedham et al., 2005).

As well as being GEFs, Vav proteins can also act as signalling adapters (Bustelo et al., 2001). Proper organisation of signalling proteins leads to the formation of a multi-protein signalling complex that regulates cellular responses to CSF-1. Purification of activated CSF-1 receptor signalling complexes (Kanagasunderam et al., 1999), and proteomic studies of tyrosine-

phosphorylated proteins in CSF-1-stimulated macrophages (Yeung and Stanley, 2003) allowed the identification of some of these proteins. These included proteins such as Src, paxillin, ERK1/2 and Vav.

ERK1/2 signaling has long been known to regulate cell survival, proliferation and differentiation. The activation of the ERK1/2 by phosphorylation on specific tyrosine and threonine residues downstream of growth factor receptors, via the adapter proteins Grb2, and activation of Raf via Sos and Ras, is well established (see review (Roux and Blenis, 2004)). In macrophages CSF-1 stimulation caused an increase in cell cycle progression, and new gene transcription (Jawarowski et al., 1999). Thus, the activation of ERK1/2 was used as a read-out of the proper formation of the CSF-1 signalling complex, and signal transduction to processes that regulate cell proliferation and gene transcription. More recently, ERK1/2 has also been reported to have a role in regulating adhesion and migration (Huang et al., 2004). Thus, ERK1/2 activation downstream of CSF-1 receptor activation could affect all aspects of macrophage responses to this cytokine.

Changes in tyrosine phosphorylation upon CSF-1 withdrawal and re-addition were not different between Wt and Vav-deficient macrophages, and the CSF-1-stimulated phosphorylation of ERK1/2, and paxillin was also the same in both Wt and Vav-deficient macrophages. This showed that the Vav proteins are not essential as signaling adapters in CSF-1-induced intracellular signaling.

Macrophage responses to CSF-1 were then analysed in a physiologically relevant context, where macrophages were exposed to CSF-1 in a gradient. Macrophages deficient in Vav proteins polarized and positively chemotaxed in a gradient of CSF-1. Furthermore, there was no evidence to suggest that Vav1/2/3<sup>-/-</sup> macrophages had any defect in the ability to chemotax toward CSF-1. It was thus shown that Vav proteins are not required for macrophage chemotaxis to CSF-1. This could be expected however, since directionality and polarization are processes largely attributed to the activities of PI3K and

Cdc42 whereas RhoA and Rac regulate the mechanics of cell contraction and protrusion respectively (Ridley et al., 2003).

Further analysis of cell migration supported this hypothesis, indicating that Vav1/2/3<sup>-/-</sup> macrophages had slightly reduced migration speed, and slightly increased migration persistence during chemotaxis. Interestingly, the absence of Vav1 in BMM has been previously reported to result in reduced migration speed (Wells et al., 2005), as also observed in this present study (Ch. 3.9). It is possible that this subtle difference in macrophage chemotaxis is a result of the contribution of Vav proteins to macrophage morphology (Chapter 3), rather than a role in the response to CSF-1. Vav1/2/3<sup>-/-</sup> BMM had an elongated morphology, which could lead to increased migration persistence, if cells are less likely to change direction during migration, as discussed in Chapter 3.

These data apparently contradict a previous study (Vedham et al., 2005) reporting the role of Vav upstream of Rac1 in BMM chemotaxis to CSF-1. This discrepancy may be a result of the different experimental methods used to study chemotaxis. In this present study, macrophage migration was analysed using a Dunn chemotaxis chamber, whereas, Vedham and colleagues used transwell assays in which cells migrate from an upper well through pores in a membrane to the bottom well containing a chemo-attractant. In addition, the study used BMM from SHIP-deficient mice. SHIP (SH2 domain-containing inositol phosphatase), hydrolyses the PI3K-product PI(3,4,5)P<sub>3</sub>. This may have led to abnormal localization of Vav (as well as other PH domain-containing GEFs) in these cells. Also, as with many other studies of Vav function, Vav-mutants were introduced into macrophages. This potentially could affect not only endogenous Vav but the functions of other GEFs too.

Taken together, previous studies suggest that Vav proteins function to activate Rac downstream of the CSF-1 receptor and here it has been shown that Vav1 and Vav3 are phosphorylated on tyrosine in response to CSF-1 stimulation in macrophages. However, with the use of Vav-deficient

macrophages, it has been demonstrated that Vav proteins are not indispensable for any CSF-1 responses tested in this study. In the absence of Vav proteins, other GEFs could activate Rac1 and regulate cytoskeletal changes upon CSF-1 stimulation. A number of studies have shown the importance of PI3K activity to CSF-1 signal transduction (Jones et al., 2003; Vedham et al., 2005). Indeed, studies have shown that Vav catalytic activity can also be regulated by phosphoinositides (Han et al., 1998). This has been implicated as the mechanism of Vav activation downstream of PI3K to Rac1 (Sakai et al., 2006; Marcoux et al., 2003). This regulation is mediated through the Vav PH domain. If phosphoinositides are a means by which Vav proteins are localized and activated, it can be hypothesized that in their absence, other PH domain-containing Rac GEFs could substitute for their activity. Indeed, the Dbl-homology domain, is invariably paired with the PH-domain, (Rossman et al., 2005). Furthermore, studies have shown that PH domain regulation of the catalytic activity of the DH domain is not limited to the Vav family of GEFs, for example PREX and Sos (Rossman et al., 2005).

Vav-deficient bone-marrow cells incubated with CSF-1 differentiate into a macrophage population, as shown by F4/80 expression (Ch. 3.4). This indirectly suggests that CSF-1 signalling to macrophage differentiation, growth and proliferation is unaffected in the absence of Vav proteins. This is further evidence that Vav proteins are not critical to the signaling events downstream of the CSF-1 receptor.

In conclusion, it appears that Vav proteins are not specifically required for CSF-1-induced macrophage signalling and morphological responses or chemotaxis.

## **5. The role of Vav proteins in adhesion-dependent spreading of macrophages**

### **5.1: Introduction**

Activation of the integrin receptors can lead to changes in cell morphology. This is exemplified during leukocyte firm adhesion to the endothelium. It is thought that leukocyte rolling on the endothelial wall of blood vessels is mediated by interactions between the leukocyte and endothelium selectins (Patel et al., 2002). Integrins on the adhered leukocyte are then ligated by endothelial cell- expressed integrin ligands such as ICAM-1 and VCAM-1, which in combination with other stimuli initiate signalling within the leukocyte that induces arrest, firm adhesion (Alon and Feigelson, 2002) and promotion of a migratory phenotype (Hood and Cheresh, 2002). Such a change in morphology is brought about essentially by reorganisation of the actin cytoskeleton.

The Rho family GTPases regulate changes in cell morphology. It has been shown that integrins signal to the Rho GTPases and in this way can induce changes in cell morphology (Ch. 1.3). Vav proteins are Rho/Rac family GEFs, as well as signalling adapters. A number of studies have highlighted their importance in integrin signalling in leukocytes. These show that in neutrophils (Gakidis et al., 2004), osteoclasts (Faccio et al., 2005) and lymphocytes (Sanchez-Martin et al., 2004), Vav proteins activate the Rho/Rac GTPases downstream of integrins. The role of Vav proteins in morphological responses and signalling initiated upon adhesion in macrophages was therefore investigated. Macrophages derived from mice lacking single isoforms of Vav (Vav1<sup>-/-</sup>, Vav2<sup>-/-</sup>, Vav3<sup>-/-</sup>) or all three isoforms (Vav1/2/3<sup>-/-</sup>) were used to carry out the investigation.



### 5.2: Vav1/2/3<sup>-/-</sup> BMM have reduced spreading on glass

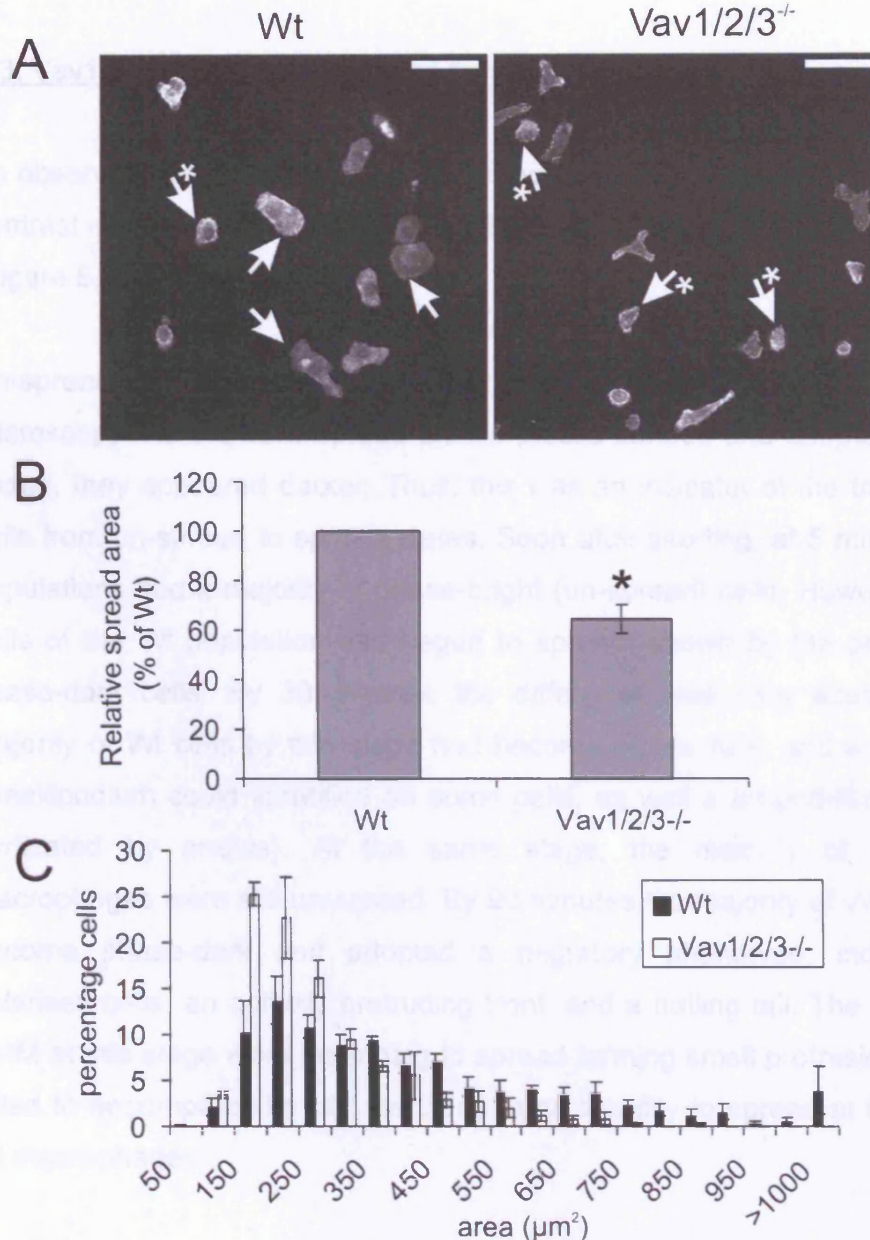
Upon contact with a substratum, cells become adherent and then spread out, becoming relatively more flat. This is achieved when signalling initiated at adhesion receptors regulates morphological changes. It has been shown that integrins are major contributors to the adhesion of cells not only *in vivo*, but also *in vitro* on glass and plastic tissue culture surfaces (Melder et al., 1991). Thus spreading of cells upon adhesion can be used to analyse their adhesion-dependent morphological responses.

To investigate the role of Vav proteins in adhesion dependent morphological changes, Wt and Vav1/2/3<sup>-/-</sup> macrophages were seeded on glass coverslips in the absence of CSF-1 and spreading was monitored over 60 minutes. Their relative spread areas were then compared. In contrast to Chapter 3, CSF-1 was not included in the medium in these experiments, as it can also stimulate cell spreading. In this way, the response of macrophages to adhesion, and no other stimulus, was studied.

Analysis of confocal images highlighted differences in spreading between the Wt and Vav1/2/3<sup>-/-</sup> BMM (Figure 5.1A). At 60 min most Wt BMM had begun to spread -indicated by the presence of lamellipodia extended from the main body of the cell (indicated by arrows). In contrast, the Vav1/2/3<sup>-/-</sup> BMM at the same timepoint had a different morphology. Though there were cells that extended lamellipodia, it appeared that a greater proportion of cells had no, or very small lamellipodia (indicated by starred arrows). These 'unspread' cells were defined as cells which were adherent, but had no detectable protrusions.

Quantification of cell spread area showed that Vav1/2/3<sup>-/-</sup> macrophages had a significantly lower mean spread area than Wt macrophages at 60 min (Figure 1B). Further analysis of the distribution of cells in different stages of spreading showed that Vav1/2/3<sup>-/-</sup> BMM had fewer cells in advanced stage of spreading (greater than 500  $\mu\text{m}^2$ ) than Wt BMM (Figure 1C). Under these conditions it

appeared that  $Vav1/2/3^{-/-}$  BMM macrophages had a defect in adhesion-dependent spreading.



**Figure 5.1: Vav1/2/3<sup>-/-</sup> BMM have a significantly smaller spread area on glass.**

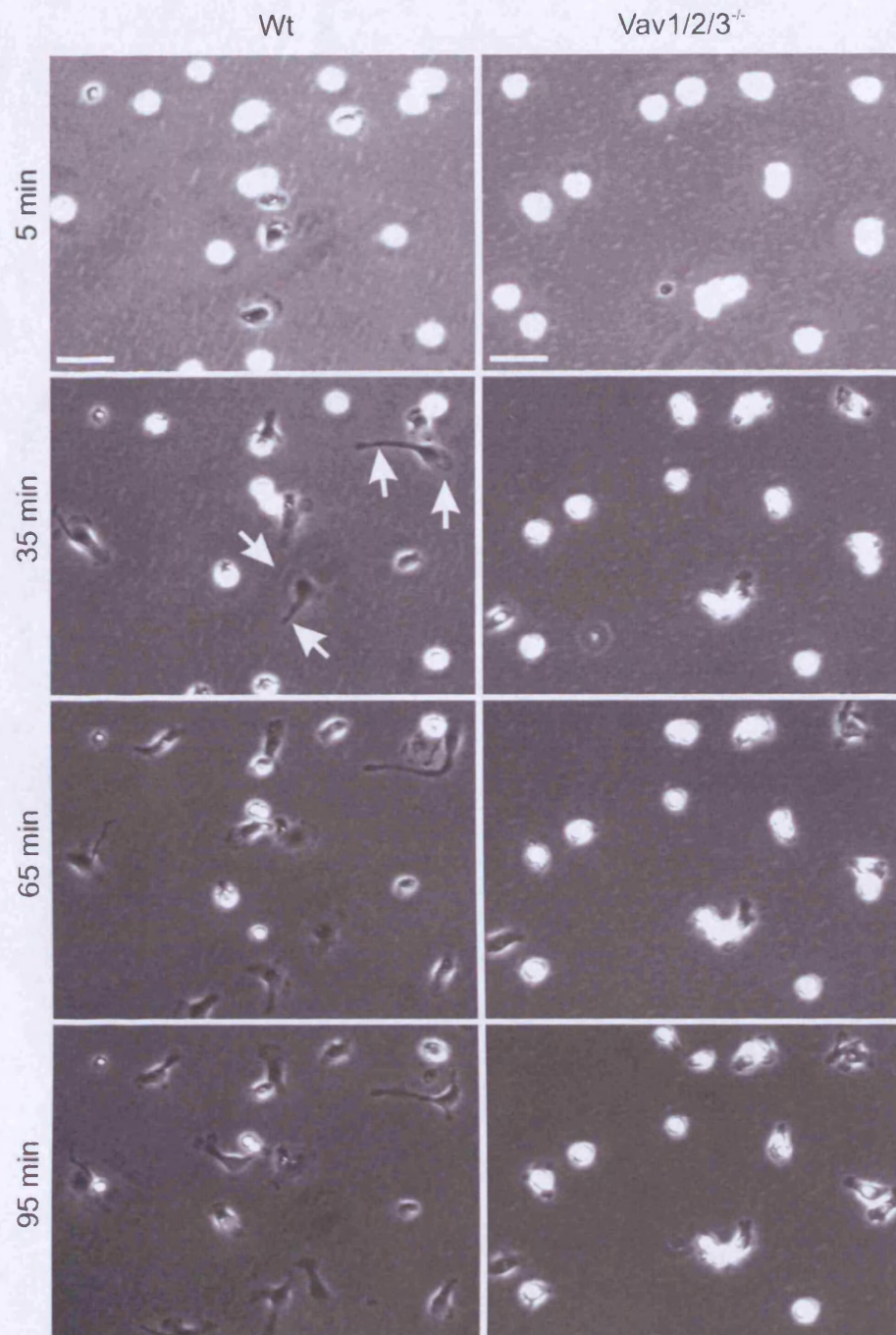
Wt and Vav1/2/3<sup>-/-</sup> BMM were resuspended in macrophage starve medium. Equal numbers of cells were seeded onto glass coverslips for 60 minutes before cells were fixed and stained for F-actin with TRITC-phalloidin (A). Arrows: lamellipodia. Starred arrows: unspread cells. Images of cells (n>100 per coverslip) were acquired by confocal microscopy (Bar=20 μm). The area of cells was determined using image analysis software and shown as the mean ±s.e.m. relative to Wt (B), or shown as percentages of cells with different areas (C). Data shown are the mean of three independent experiments. A Student's t-test was used to determine statistical significance (\*P<0.05, Vav1/2/3<sup>-/-</sup> compared to Wt).

### 5.3: Vav1/2/3<sup>-/-</sup> BMM have reduced spreading on plastic

To observe the dynamics of cell spreading upon adhesion, time-lapse phase-contrast images of Wt and Vav1/2/3<sup>-/-</sup> BMM adhering on plastic were recorded (Figure 5.2, movies 14 and 15).

Un-spread/rounded cells appeared intensely bright by phase-contrast microscopy. As the cells spread on the plastic surface and adopted a flatter shape, they appeared darker. Thus, this was an indicator of the transition of cells from un-spread to spread states. Soon after seeding, at 5 minutes both populations had a majority of phase-bright (un-spread) cells. However, a few cells of the Wt population had begun to spread, shown by the presence of phase-dark cells. By 30 minutes the difference was very apparent. The majority of Wt cells by this stage had become phase-dark, and a protruding lamellipodium could be identified on some cells, as well as a uropod-like structure (indicated by arrows). At the same stage, the majority of Vav1/2/3<sup>-/-</sup> macrophages were still un-spread. By 90 minutes the majority of Wt cells had become phase-dark and adopted a migratory phenotype, indicative of polarised cells: an actively protruding front, and a trailing tail. The Vav1/2/3<sup>-/-</sup> BMM at this stage were beginning to spread forming small protrusions, which failed to encompass the cell, resulting in an inability to spread at the rate of Wt macrophages.

Macrophages lacking Vav proteins therefore had a defect in spreading on both glass and plastic tissue culture surfaces. Though time-lapse analysis of spreading was carried out using a plastic surface, it would be interesting to also observe spreading on glass.



**Figure 5.2: Vav1/2/3<sup>-/-</sup> BMM have reduced spreading upon adhesion to plastic.** Wt and Vav1/2/3<sup>-/-</sup> BMM were resuspended in macrophage starve medium. Cells were seeded onto plastic tissue culture dishes. Phase-contrast images of cells were then acquired at 1 frame/min for 95 min. A montage of frames at 5, 35, 65 and 95 minutes after seeding is shown here (movies 14 and 15). Bar=40  $\mu$ m. Frames from a representative movie of 4 independent experiments is shown.



#### 5.4: BMM deficient for Vav1, Vav2 or Vav3 spread normally on glass

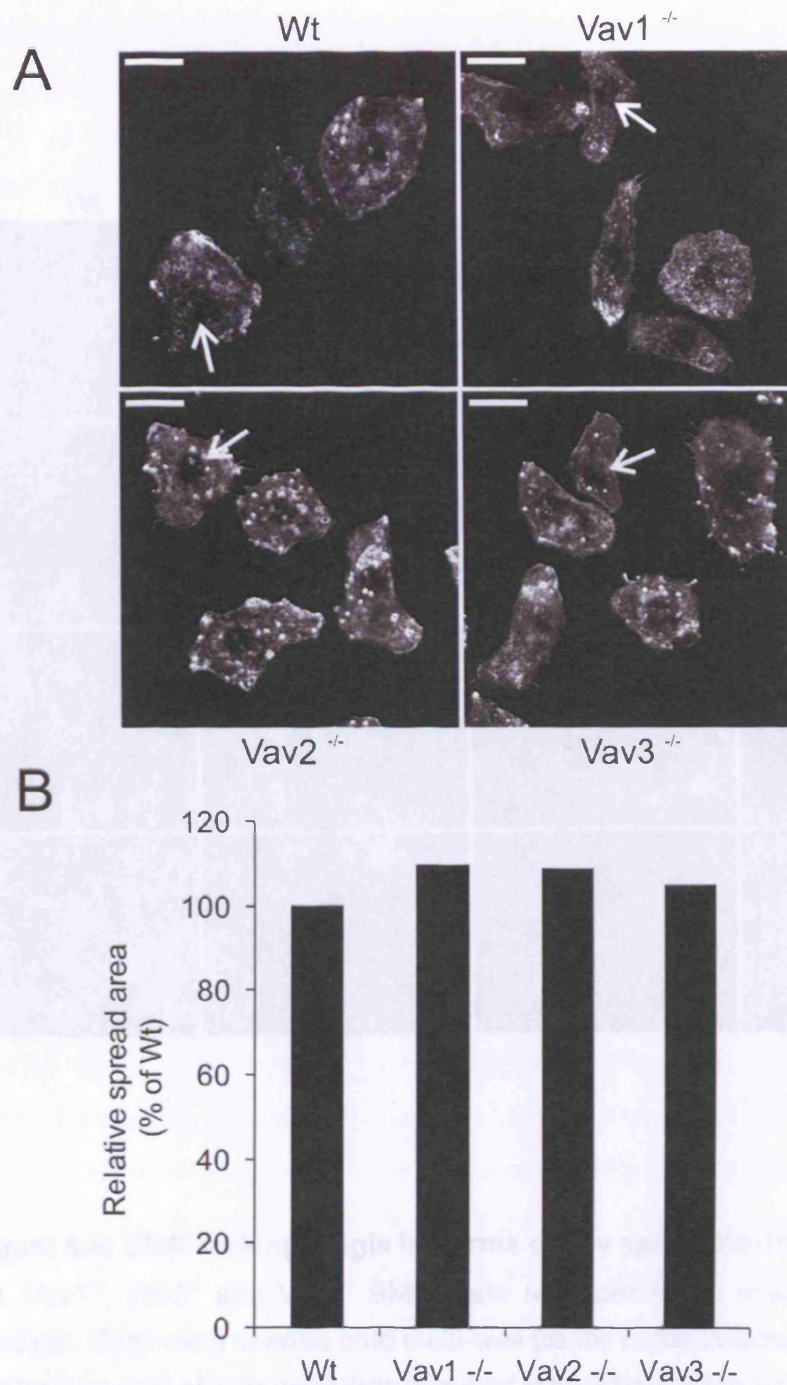
To determine if the spreading defect was due to the absence of any single Vav isoform, the spreading analyses were repeated using macrophages derived from mice lacking only single isoforms of the Vav proteins.

Analysis of images of spreading Wt BMM and BMM lacking single isoforms of Vav 60 min after seeding on coverslips revealed that all the populations had adopted a similar morphology (Figure 5.3A). BMM of all populations had formed protrusions that encircled the nuclear region of the cell (which appeared as a region of less intense F-actin staining in the centre of the cell, indicated by arrows), indicating the extent of cell spreading. Interestingly, all populations of spread BMM had puncta of F-actin in the cell body, which could indicate the formation of adhesions structures such as podosomes. Quantitative analysis of cell spread area showed that Vav-deficient macrophage populations consistently had a spread area comparable to the Wt population at the same time after seeding (Figure 5.3B).

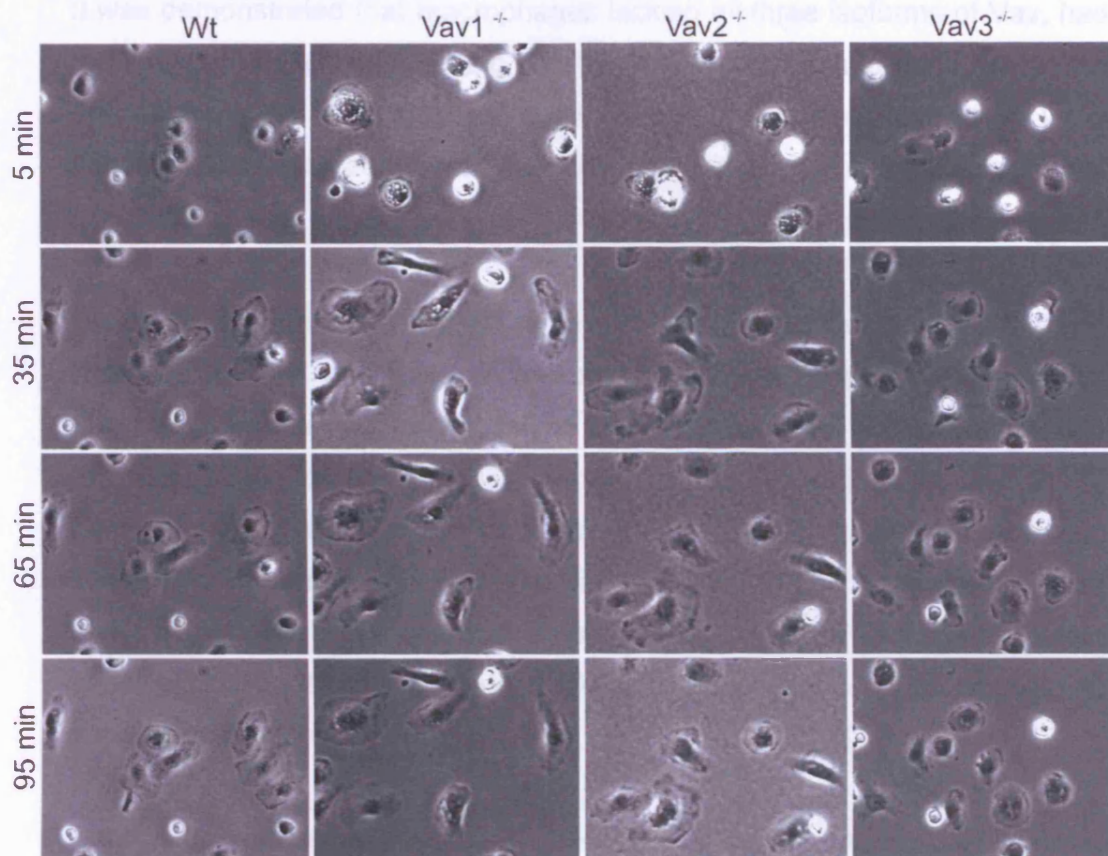
This indicated that in the absence of single isoforms of Vav, BMM made a normal morphological response to adhesion. The absence of any single isoform of Vav was therefore not responsible for the deficiency in adhesion-dependent spreading observed in Vav1/2/3<sup>-/-</sup> BMM.

#### 5.5: BMM deficient for Vav1, Vav2 or Vav3 spread normally on plastic

To confirm the normal spreading response of the macrophages lacking only single isoforms of Vav, spreading Wt BMM and BMM lacking single isoforms of Vav were observed by phase-contrast time-lapse microscopy. All three Vav-deficient populations displayed normal spreading on plastic comparable to Wt BMM (Figure 5.4 and movies 16 to 19). BMM were observed to extend localised protrusions (5 to 30 minutes) that over time had spread and encircled the whole cell (65 minutes). At advanced stages (beyond 60 minutes), ruffling was observed around the peripheral regions of the cell.



**Figure 5.3: BMM lacking single isoforms of Vav spread normally on glass.** Wt, Vav1<sup>-/-</sup>, Vav2<sup>-/-</sup> and Vav3<sup>-/-</sup> BMM were resuspended in macrophage starve medium. Equal numbers of cells were seeded onto glass coverslips for 60 minutes before cells were fixed and stained for F-actin with TRITC-phalloidin. Images of cells ( $n > 100$  per coverslip) were acquired by confocal microscopy (A). Arrows: nuclear region of cell. Bar=20  $\mu$ m. The area of cells was determined using image analysis software and shown here as the mean relative to Wt (B). Data shown here are the mean of two independent experiments.



**Figure 5.4: BMM lacking single isoforms of Vav spread normally on plastic.**

Wt, Vav1<sup>-/-</sup>, Vav2<sup>-/-</sup> and Vav3<sup>-/-</sup> BMM were resuspended in macrophage starve medium. Cells were seeded onto multi-well plastic tissue culture dishes. Phase-contrast images of cells were then acquired in parallel at 1 frame/min for 95 min. A montage of frames at 5, 35, 65 and 95 minutes after seeding is shown (movies 16, 17, 18 and 19). Frames are from representative movies of 2 independent experiments.



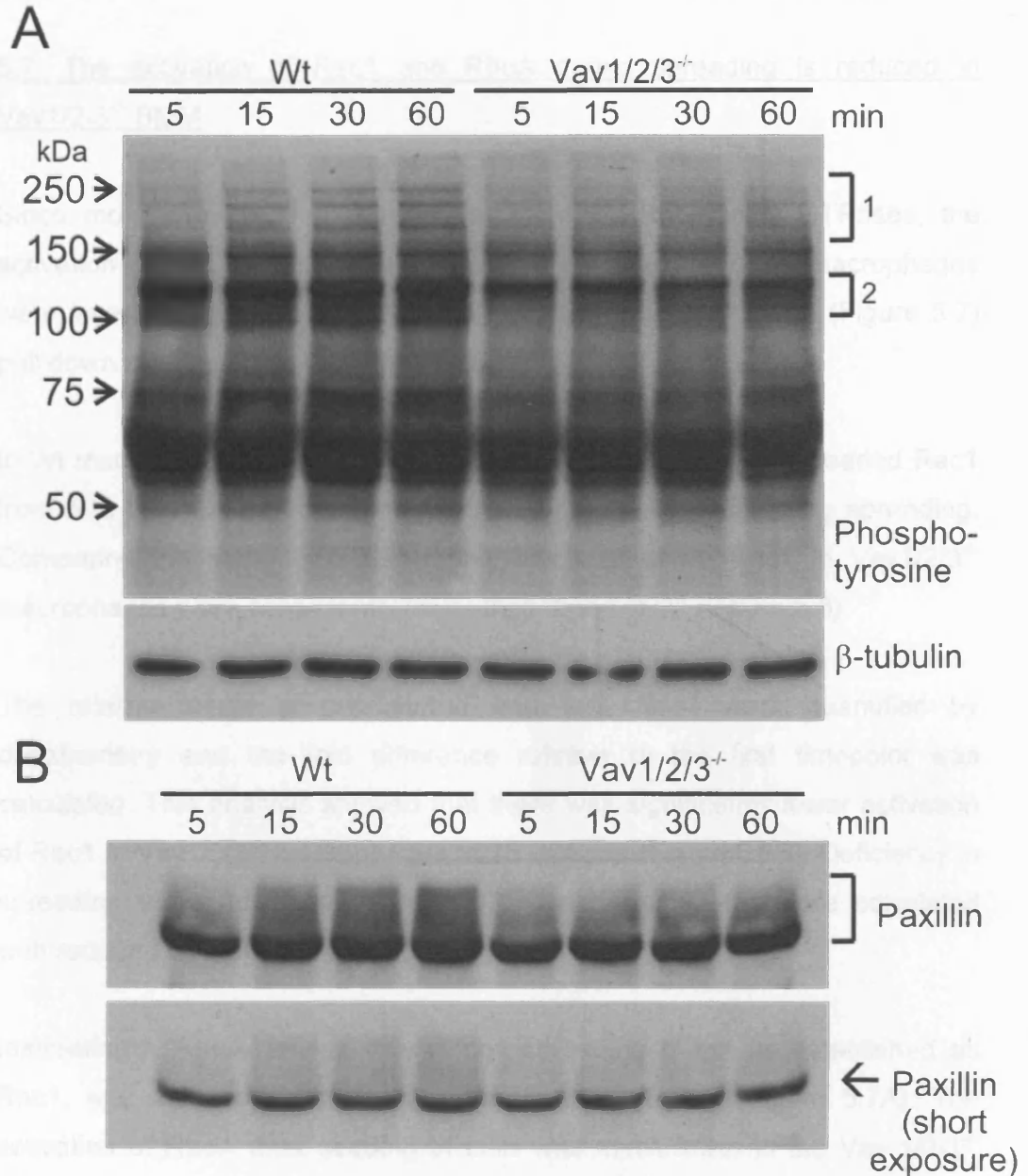
### 5.6: Tyrosine and paxillin phosphorylation in response to adhesion are reduced in Vav1/2/3<sup>-/-</sup> BMM

It was demonstrated that macrophages lacking all three isoforms of Vav, had reduced spreading after seeding onto glass and plastic. In order to study how the absence of Vav proteins could result in reduced spreading upon adhesion, adhesion-dependent signalling in Wt and Vav1/2/3<sup>-/-</sup> macrophages was compared.

Proteins recruited to integrin signalling complexes include the tyrosine kinases Src, FAK and Pyk2 (in leukocytes) and the adapter protein paxillin (Turner, 2000). Paxillin also becomes phosphorylated on tyrosine and serine residues during spreading (Bellis et al., 1997; Richardson et al., 1997). Furthermore, phosphorylation at certain serine residues causes an alteration in the electrophoretic mobility of paxillin (Cai et al., 2006). Thus, the progress of spreading could be followed by monitoring changes in cellular protein tyrosine phosphorylation (Figure 5.5A) and the phosphorylation of proteins such as paxillin (Figure 5.5B).

During the course of spreading, there was a steady increase in level of tyrosine phosphorylation in Wt macrophages (figure 5.5A). In particular, proteins above an apparent molecular weight of 150 kDa became increasingly tyrosine phosphorylated from 5 to 60 minutes, whereas this was not evident in Vav1/2/3<sup>-/-</sup> macrophages (Figure 5A, bracket 1). There was also an increase in the tyrosine phosphorylation of proteins of apparent molecular weights between 100 and 120 kDa in Wt BMM during spreading. In the corresponding positions in Vav1/2/3<sup>-/-</sup> macrophages this was not to the same extent, though there was a slight increase over time (Figure 5A, bracket 2). Interestingly, a protein of apparent molecular weight just below 100 kDa was increasingly tyrosine phosphorylated from 5 to 60 minutes of spreading in Wt BMM. This band, likely to be one of the Vav proteins, was not detected in Vav1/2/3<sup>-/-</sup> BMM.

Paxillin phosphorylation during spreading in Wt and *Vav1/2/3<sup>-/-</sup>* BMM was followed by the level of paxillin shifted to lower mobility (serine-phosphorylated) forms (Figure 5.5B). In Wt macrophages at 5 minutes after seeding, the paxillin phosphorylation shift was hardly detected. However, as the cells became more spread, the level of the lower mobility forms of paxillin increased. In *Vav1/2/3<sup>-/-</sup>* macrophages, the phosphorylation of paxillin at 5 minutes was comparable to Wt cells at the same timepoint. However, the paxillin lower mobility forms did not increase to the extent of Wt macrophages during spreading to 60 minutes. Thus, reduced protein tyrosine phosphorylation and phosphorylation of paxillin correlate with reduced spreading of BMM lacking all three Vav proteins.



**Figure 5.5: Tyrosine and paxillin phosphorylation in response to adhesion is reduced in Vav1/2/3<sup>-/-</sup> BMM.** Wt and Vav1/2/3<sup>-/-</sup> BMM were resuspended in macrophage starve medium. Equal numbers of cells were seeded onto plastic tissue culture dishes for the indicated times before being solubilised in lysis buffer. Proteins were resolved by 4-12% gradient SDS-PAGE. Changes in protein tyrosine phosphorylation and paxillin phosphorylation were monitored by western blotting using anti-phosphotyrosine (4G10) (A), or paxillin (B) antibodies respectively (β-tubulin, and a short exposure of paxillin were used as loading controls respectively). Brackets: tyrosine phosphorylated proteins at 150 to 250 kDa (1) and 100 to 120 kDa (2). Shown here are representative blots from 3 independent experiments.

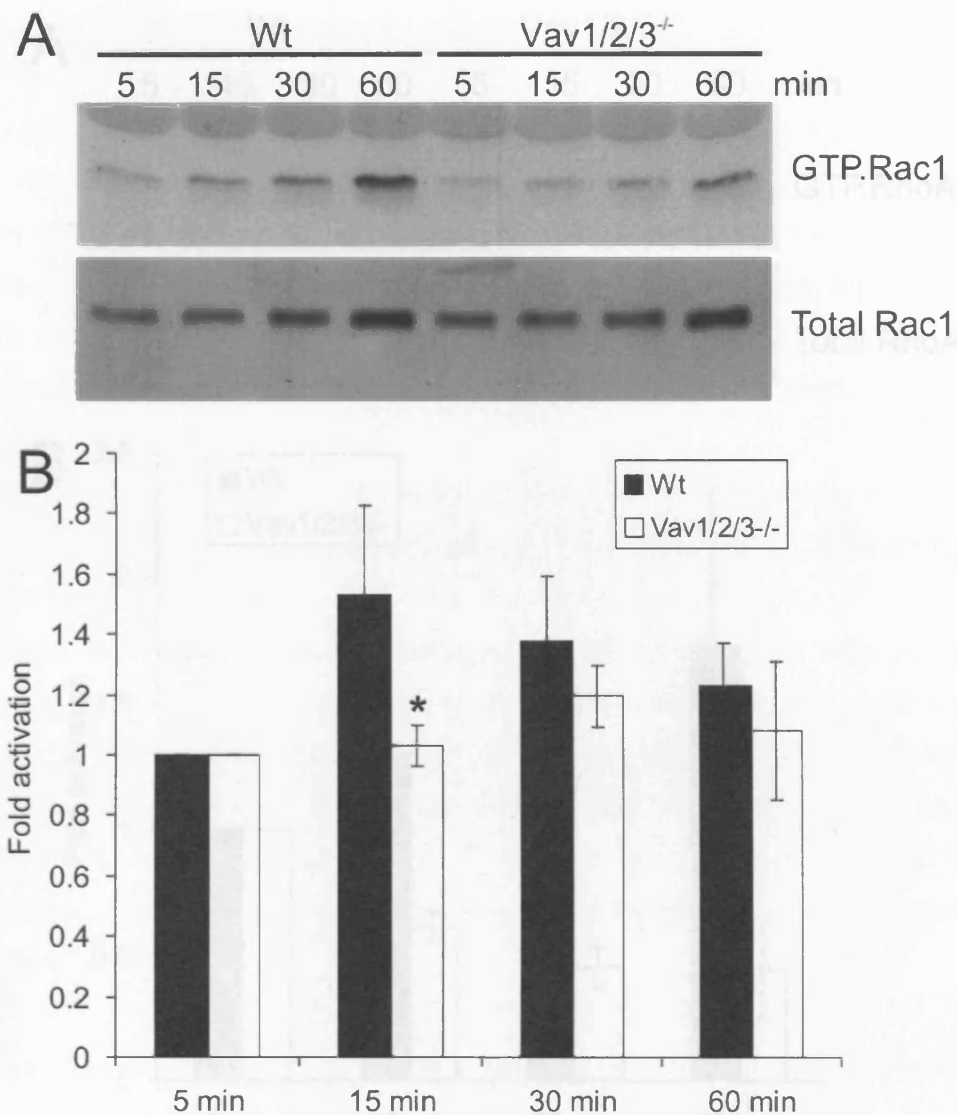
### 5.7: The activation of Rac1 and RhoA during spreading is reduced in Vav1/2/3<sup>-/-</sup> BMM

Since morphological changes are regulated by Rho family GTPases, the activation of Rac1 and RhoA in spreading Wt and Vav1/2/3<sup>-/-</sup> macrophages were investigated using GTP.Rac1 (Figure 5.6) and GTP.RhoA (Figure 5.7) pull down assays.

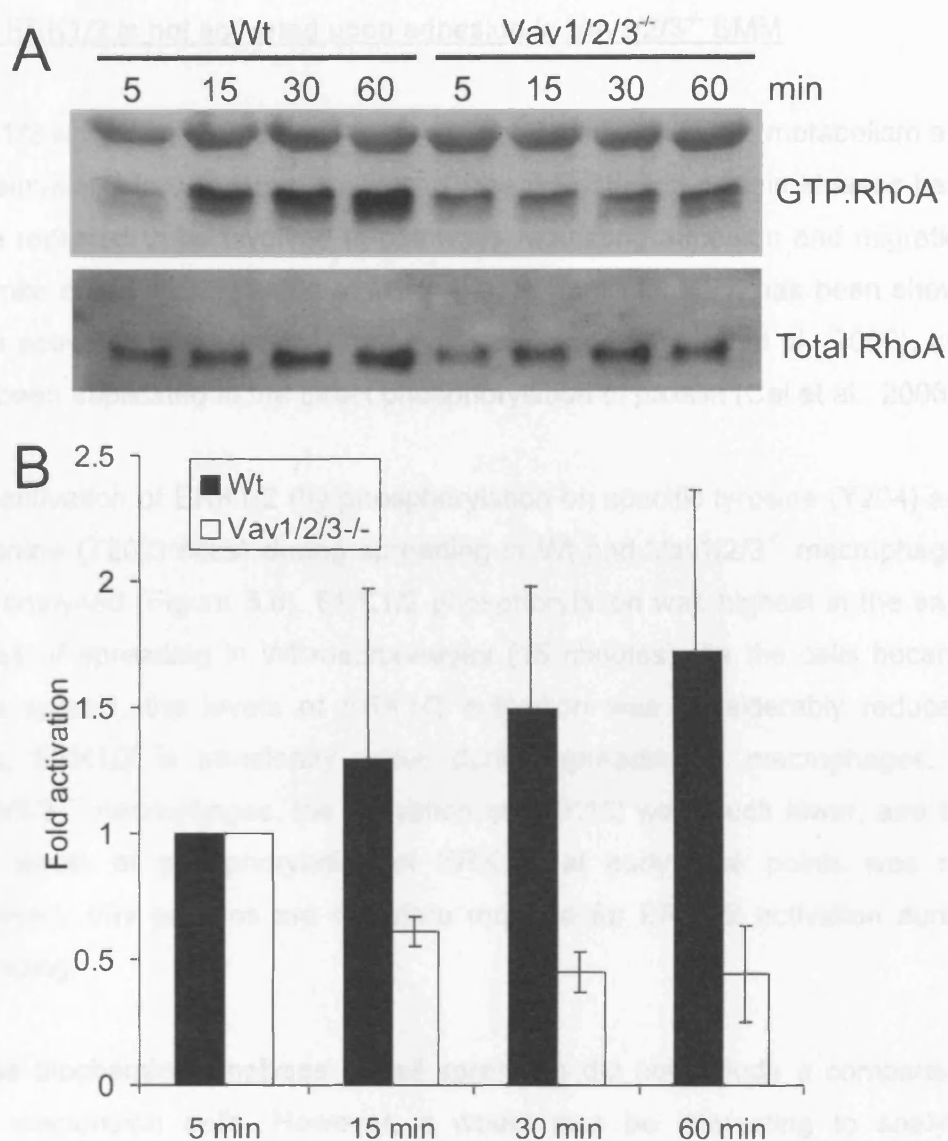
In Wt macrophages there was an increase in the levels of GTP-loaded Rac1 from 5 to 60 minutes compared to the total Rac1 in WCL during spreading. Comparing the same time-points, the levels of active Rac1 in Vav1/2/3<sup>-/-</sup> macrophages were consistently lower than those of Wt (Figure 5.6).

The relative levels of precipitated and total Rac1 were quantified by densitometry and the fold difference relative to the first timepoint was calculated. This analysis showed that there was significantly lower activation of Rac1 in Vav1/2/3<sup>-/-</sup> macrophages at 15 minutes (Figure 5.6B). Deficiency in spreading upon adhesion in Vav1/2/3<sup>-/-</sup> macrophages therefore correlated with reduced activation of Rac1.

Interestingly, RhoA, whose role in cell spreading is not as established as Rac1, was also activated during spreading in Wt BMM (figure 5.7A). The activation of RhoA after seeding of cells was much lower in the Vav 1/2/3<sup>-/-</sup> macrophages from 15 to 60 minutes. Quantification of fold-activation relative to the 5 minute time-point, showed that whereas in Wt macrophages, RhoA activity tended to increase upon adhesion, in Vav1/2/3<sup>-/-</sup> macrophages, its activity was either unchanged, or decreased during spreading (Figure 5.7B). However, the high variation in fold-activation in different experiments meant that no significant differences were detected. Reduced spreading upon adhesion in Vav1/2/3<sup>-/-</sup> macrophages therefore correlated with reduced activation of both Rac1 and RhoA.



**Figure 5.6: The activation of Rac1 is reduced during spreading of Vav1/2/3<sup>-/-</sup> BMM.** Wt and Vav1/2/3<sup>-/-</sup> BMM were resuspended in macrophage starve medium. Equal numbers of cells were seeded onto plastic tissue culture dishes for the indicated times, before being solubilised. Lysates were then incubated with glutathione sepharose beads coupled to GST-PAK1-PBD, to precipitate GTP-bound Rac1. Precipitates and whole cell lysates (WCL) were resolved by 12% SDS-PAGE. (A) Rac1 levels in precipitates (GTP.Rac1) and WCL (Total Rac1) were compared by western blotting with an anti-Rac1 antibody followed by densitometry. (B) GTP.Rac1 levels were normalised to the total Rac1, and fold activation determined relative to the earliest time-point, 5 min. (A) is representative of three independent experiments and (B) is the mean of these experiments  $\pm$  s.e.m. Statistical significance was determined by a Student's t-test (\* $P < 0.05$ , Vav1/2/3<sup>-/-</sup> compared to Wt at each timepoint).



**Figure 5.7: The activation of RhoA is reduced during spreading of Vav1/2/3<sup>-/-</sup> BMM.** Wt and Vav1/2/3<sup>-/-</sup> BMM were resuspended in macrophage starve medium. Equal numbers of cells were seeded onto plastic tissue culture dishes for the indicated times before being solubilised. Lysates were then incubated with glutathione sepharose beads coupled to GST-Rhotekin-RBD, to precipitate GTP.RhoA. Precipitates and WCL were resolved by 12% SDS-PAGE. (A) RhoA levels from precipitates (GTP.RhoA) and WCL (total RhoA) were compared by western blotting with an anti-RhoA antibody followed by densitometry. (B) GTP.RhoA levels were normalised to the total RhoA levels and fold activation determined relative to the earliest timepoint, 5 min. (A) is representative of three independent experiments and (B) is the mean of these experiments  $\pm$  s.e.m.

### 5.8: ERK1/2 is not activated upon adhesion in Vav1/2/3<sup>-/-</sup> BMM

ERK1/2 signalling regulates processes such as the cell cycle, metabolism and cell survival. More recently however, these well-studied protein kinases have been reported to be involved in pathways regulating adhesion and migration (Klemke et al., 1997; Huang et al., 2004). In particular ERK has been shown to be activated downstream of integrin activation (Fincham et al., 2000), and has been implicated in the direct phosphorylation of paxillin (Cai et al., 2006).

The activation of ERK1/2 (by phosphorylation on specific tyrosine (Y204) and threonine (T202) sites) during spreading in Wt and Vav1/2/3<sup>-/-</sup> macrophages was analysed (Figure 5.8). ERK1/2 phosphorylation was highest in the early stages of spreading in Wt macrophages (15 minutes). As the cells became more spread, the levels of ERK1/2 activation was considerably reduced. Thus, ERK1/2 is transiently active during spreading in macrophages. In Vav1/2/3<sup>-/-</sup> macrophages, the activation of ERK1/2 was much lower, and the high levels of phosphorylation of ERK1/2 at early time points was not observed. Vav proteins are therefore required for ERK1/2 activation during spreading.

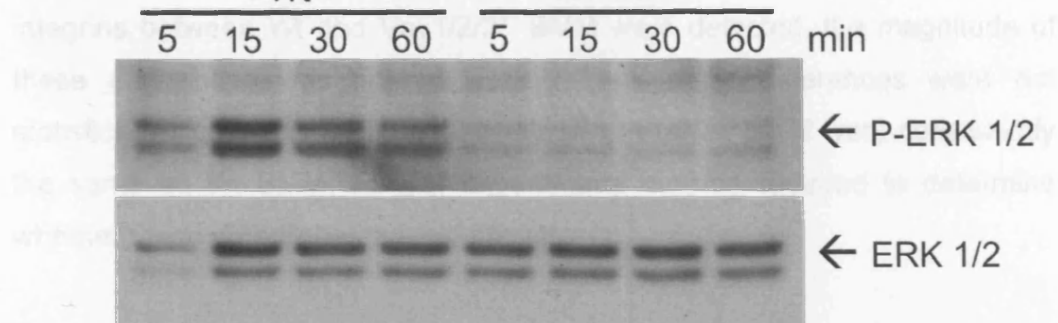
These biochemical analyses of cell spreading did not include a comparison with suspension cells. However, it would also be interesting to analyse changes that occur upon adhesion, and the possible role of Vav proteins at this step.

Although the adhesion of Wt and Vav1/2/3<sup>-/-</sup> BMM was not directly compared, the experimental evidence suggested that Vav1/2/3<sup>-/-</sup> BMM were not less adherent than Wt. Lysates of both populations after seeding on plastic for different times consistently had equal amounts of total protein at each time-point, as observed by western blotting (Figures 5.5, 5.6 and 5.7). This suggests that both BMM populations adhered to the surface at similar rates.

5.8 Vav1/2/3<sup>-/-</sup> BMM adhesion levels are not significantly different from Vav1/2/3<sup>+/+</sup> BMM

To investigate the possibility that reduced spreading of Vav1/2/3<sup>-/-</sup> BMM was due to an altered level of surface integrins, the surface expression of  $\beta$ -integrins was compared to Vav1/2/3<sup>+/+</sup> BMM by flow cytometry (Figure 5.9).

Vav1/2/3<sup>-/-</sup> BMM consistently had lower expression of  $\beta$ 2 integrin, and higher expression of  $\beta$ 3. Although the expression of  $\beta$ 2 and  $\beta$ 3



**Figure 5.8: ERK1/2 is not activated in response to adhesion in Vav1/2/3<sup>-/-</sup> BMM.** Wt and Vav1/2/3<sup>-/-</sup> BMM were resuspended in macrophage starve medium. Equal numbers of cells were seeded onto plastic tissue culture dishes for the indicated times, before being solubilised in lysis buffer. Proteins were resolved by 4-12% gradient SDS-PAGE. The activation of ERK1/2 was monitored by western blotting with polyclonal antibodies directed towards phospho-Thr202/Tyr204 (upper blot). Membranes were stripped and re-probed for ERK1/2 (lower blot). Blots are representative of 3 independent experiments.

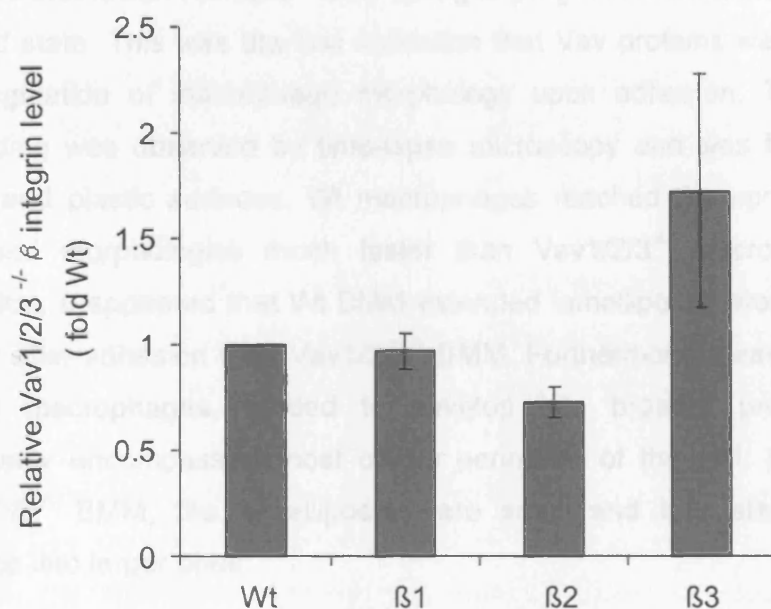


### 5.9: Vav1/2/3<sup>-/-</sup> BMM $\beta$ -integrin levels are not significantly different from Wt BMM

To investigate the possibility that reduced spreading of Vav1/2/3<sup>-/-</sup> BMMs was due to an altered level of surface integrins, their surface expression of  $\beta$ -integrins was compared to Wt BMM by flow cytometry (Figure 5.9).

Vav1/2/3<sup>-/-</sup> BMM consistently had lower expression of  $\beta 2$  integrin, and higher expression of  $\beta 3$  integrin. Although differences in expression of  $\beta 2$  and  $\beta 3$  integrins between Wt and Vav1/2/3<sup>-/-</sup> BMM were detected, the magnitude of these differences was not consistent. Thus these differences were not statistically significant.  $\beta 1$  integrin levels of Vav1/2/3<sup>-/-</sup> BMM were consistently the same as Wt BMM. Further experiments may be required to determine whether these differences are significant.

Altered levels of  $\beta$  integrins expressed by Vav1/2/3<sup>-/-</sup> BMM could therefore also contribute to reduced adhesion-dependent spreading.



**Figure 5.9:  $\beta$  integrin expression on wt and Vav1/2/3<sup>-/-</sup> BMM is not significantly different.** Equal numbers of Wt and Vav1/2/3<sup>-/-</sup> BMM were washed in PBS and resuspended in FACS buffer. BMM were then incubated with fluorophore-conjugated antibodies towards  $\beta 1$  (CD29),  $\beta 2$  (CD18),  $\beta 3$  (CD61) or no stain as a control. BMM were washed several times and their levels of surface expressed  $\beta$  integrin analysed by flow cytometry. Mean fluorescence values were normalised to the no-stain controls, and the  $\beta$  integrin levels of Vav1/2/3<sup>-/-</sup> BMM relative to Wt were calculated. Shown here are the mean fold changes relative to Wt BMM expression for three independent experiments  $\pm$  s.e.m.

### **5.10 Conclusions and Discussion**

4The contribution of Vav proteins in macrophage morphological responses and signalling downstream of adhesion was investigated.

It was first observed that macrophages lacking all three isoforms of Vav had a reduced spread area compared to Wt BMM when seeded on glass coverslips. Further analysis of the distribution of spread areas within the two populations showed that fewer Vav1/2/3<sup>-/-</sup> macrophages progressed from the un-spread to spread state. This was the first indication that Vav proteins were involved in the regulation of macrophage morphology upon adhesion. This defect in spreading was observed by time-lapse microscopy and was found on both glass and plastic surfaces. Wt macrophages reached the spread and then polarised morphologies much faster than Vav1/2/3<sup>-/-</sup> macrophages upon adhesion. It appeared that Wt BMM extended lamellipodial protrusions much earlier after adhesion than Vav1/2/3<sup>-/-</sup> BMM. Furthermore, these lamellipodia, in Wt macrophages, tended to develop into broader protrusions that eventually encompassed most of the perimeter of the cell. In contrast, in Vav1/2/3<sup>-/-</sup> BMM, the lamellipodia were small and less stable, failing to develop into larger ones.

Unlike Vav1/2/3<sup>-/-</sup> macrophages, those lacking only single isoforms of Vav had similar spreading properties to Wt macrophages. This is in contrast with previous studies that report isoform-specific roles of Vav proteins in adhesion-mediated processes in other cell types. For example, Vav1 was shown to regulate Rac1 downstream of the  $\beta_2$  integrin receptor LFA-1 in T-cells (Sanchez-Martin et al., 2004). Vav1 was reported to be required for the activation of RhoA downstream of  $\beta_3$  integrin ligation in an immature leukocyte cell line (Gao et al., 2005), and Vav2 for the spreading of fibroblasts on fibronectin (Marignani and Carpenter, 2001). However, the role of individual Vav isoforms in these pathways was investigated by expression of exogenous Wt or mutant forms of Vav family members. Over-expression of single Vav isoforms could affect the functions of the endogenously expressed Vav proteins, masking redundant functions they may have.

In contrast, studies carried out using other cell types isolated from gene-targeted mice report redundant functions of the family members. Vav1 and Vav3 were both required for neutrophil functions in response to  $\beta_2$  integrin activation (Gakidis et al., 2004) and have redundant roles in signalling stimulated by collagen in platelets (Pearce et al., 2004). Thus it is evident that gene knock-out can highlight functional redundancy between Vav proteins, where other approaches may not. Similarly, in BMM, defects in spreading upon adhesion appeared to be due to the absence of all three Vav family members. Although evidence from the literature suggests that it is likely to be due the redundant functions of Vav1 and Vav3. It would therefore be interesting to study the spreading properties of Vav double-deficient BMM.

The reduced spreading of Vav1/2/3<sup>-/-</sup> macrophages was indicative of a defect in the membrane protrusive machinery of the cells. As it has been described (Ch. 1.1), cell protrusion is driven by the organised polymerisation of actin into bundles (resulting in filopodia), or branched networks (resulting in lamellipodia). Cell spreading in response to adhesion occurs when sheet-like lamellipodia are extended from the cell in response to cell contact with the surface. Vav1/2/3<sup>-/-</sup> macrophages had reduced levels of active Rac1 during spreading compared to Wt macrophages. Both Rac1 and Cdc42 have been implicated in the formation of membrane protrusions such as ruffles, lamellipodia and filopodia. Detailed analyses of the regulation of different actin structures have shown that Rac activity is associated with the formation of lamellipodia, via Arp2/3 complex activators WAVE and Ena/VASP (Miki et al., 1998; Nakagawa et al., 2001). As discussed (Ch. 1.3), the integrin adhesion receptors can activate the Rho GTPases to induce morphological changes. And it has been shown that integrin-mediated adhesion can activate Rac1, leading to production of stable lamellipodia (Choma et al., 2004). Vav proteins may therefore be required for the Rac1-dependent reorganisation of the actin cytoskeleton, driving cell protrusion in response to adhesion receptor activation.

Interestingly, activation of RhoA during spreading was also observed in Wt BMM, which was consistently lower in *Vav1/2/3<sup>-/-</sup>* BMM. The activation of RhoA during spreading has been reported previously in the K562 leukocyte cell line (Gao et al., 2005). In contrast, during the spreading of fibroblasts on fibronectin, a transient reduction of RhoA activity was detected that was possibly mediated via FAK (Ren et al., 2000). Such a reduction of RhoA activity was not observed in the spreading of macrophages in this investigation. However, it may be that the RhoA activation kinetics upon adhesion are different in macrophages. Alternatively, it could also be that the suppression of RhoA activity does occur in macrophages during spreading, but could not be captured in the timecourse used in this study.

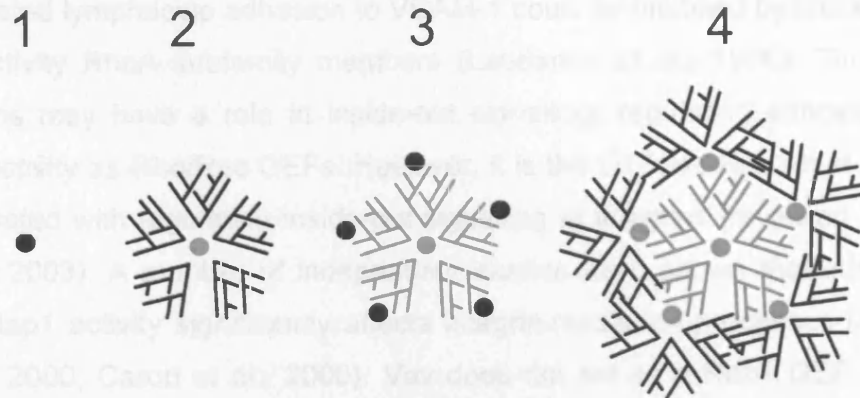
It has been suggested that the activities of Rac and RhoA are reciprocally regulated within the same locality so that they are not active in the same region of the cell (Rottner et al., 1999; Sander et al., 1999). This has been used as a basis for models on the establishment of a migratory morphology; where localised Rac activity regulates formation of the cell leading edge, and RhoA, the tail (Ch. 1.2). Images of macrophages spreading on glass indicate that initially cells spread and adopt a circular, non-polarised morphology. However, the Rac1 and RhoA pull-down assays were conducted using cells adhered to plastic dishes. Movies of Wt macrophages spreading on plastic show that at any one timepoint, there is a mixed population of cells with circular or polarised morphologies, and of these some could be seen to be migrating. In this way, it can be postulated that in macrophages spreading as polarised -and often migrating- cells, activation of both Rac1 and RhoA could be present in these cells simultaneously, and regulating the morphological changes.

RhoA may also be activated during spreading because it has an important role in cell protrusion. RhoA activity has been shown to localise in areas of lamellipodium formation, as well as in peripheral ruffles in migrating fibroblasts (Pertz et al., 2006). Detailed analysis of cell protrusion dynamics during spreading have shown that they comprise of protrusion, adhesion and retraction events (Giannone et al., 2006). And interestingly, the simultaneous

but distinctly localised activities of RhoA and Cdc42 were required in the F-actin reorganisation leading to closure of xenopus oocyte wounds (Benink and Bement, 2005). Thus, it could be that cell protrusion requires the localised activities of both Rac and RhoA.

RhoA and Rac-mediated regulation of the actin cytoskeleton could contribute to cell spreading in more than one way. Rho family GTPase regulation of the cytoskeleton is well established. However, there is also evidence to support important roles of these proteins in regulating cell adhesions. Cytochalasin B is a mycotoxin that blocks actin polymerisation and disrupts the actin cytoskeleton. Cytochalasin B treatment of adherent macrophages has been shown to cause their detachment from the culture surface (Helentjaris et al., 1976). Actin polymerisation thus has an essential role in maintaining macrophage adhesions as well as morphology. Indeed, macrophages, osteoclasts and other monocyte-lineage cells can form adhesions to the ECM through structures called podosomes (Linder and Kopp, 2005; Evans and Matsudaira, 2006). These are different from other adhesion structures such as focal contacts or focal adhesions. Uniquely, podosomes have a central core of bundled actin filaments surrounded by proteins such as paxillin and Arp2/3 complex. There is evidence to suggest that both Rac and RhoA activities influence cellular adhesion complexes (Rottner et al., 1999), where Rac was shown to have a role in promoting new adhesion formation, and RhoA in the maturation of existing ones. The regulation of podosome-like structures by Rho GTPases has been previously demonstrated in endothelial cells (Osiak et al., 2005) and dendritic cells (Burns et al., 2001).

Regulators of F-actin, Rac1 and RhoA, could therefore affect cell spreading in macrophages through regulation of cell protrusion as well as the adhesions that stabilise them. As Rho/Rac GEFs, these are possible mechanisms through which Vav proteins can promote cell spreading.



**Figure 5.10. A Model for positive feedback mechanisms during cell spreading.** 1. The cell in suspension makes an initial contact with the substrate. 2. Signalling initiated at the adhesion site stimulates membrane protrusion via actin polymerisation. 3. Cell protrusion allows further adhesions to be formed. 4. New adhesions stimulate further membrane protrusion.

In a simplified model of cell spreading (Figure 5.10), initial adhesion stimulates protrusion, which allows further adhesions to be formed, and so on. Inhibition of either adhesion or protrusion processes can affect cell spreading. Specifically, it is possible that the spreading defects caused by the absence of Vavs are not due to abnormal cell protrusion, but due to defects in the formation and signalling from adhesions. Though there were variations across experiments no statistically significant differences in  $\beta$ -integrin expression were observed between Wt and Vav1/2/3<sup>-/-</sup> macrophage populations. This suggests that Vav1/2/3<sup>-/-</sup> BMM have the same capacity for adhesion as Wt BMM, and thus their ability to *initiate* signalling via integrins should not be impaired.

It is well-known that integrins are also subject to regulation from within the cell, by so called inside-out signalling (van der Flier and Sonnenberg, 2001). Signals from within the cells, from growth factor receptors or chemo-attractants can regulate integrin affinity for their extracellular ligand, and their clustering (avidity). Evidence suggests that this regulation could involve Rho GTPases. It was shown that stimulation of lymphocytes with the chemokine CXCL12 activated Vav1 and Rac1, and increased their  $\alpha 4 \beta 1$ -mediated

adhesion (Garcia-Bernal et al., 2005). It was also shown that fMLP or IL-8-stimulated lymphocyte adhesion to VCAM-1 could be inhibited by blocking the activity of RhoA subfamily members (Laudanna et al., 1996). Thus, Vav proteins may have a role in inside-out signalling, regulating adhesions via their activity as Rho/Rac GEFs. However, it is the GTPase Rap1 that is most associated with regulating inside-out signalling of integrins (reviewed in Bos et al., 2003). A number of independent studies have shown that interfering with Rap1 activity significantly affects integrin-mediated processes (Katagiri et al., 2000; Caron et al., 2000). Vav does not act as a Rap1 GEF, rather, GEFs such as Vav require Rap1 activity for their correct localisation (Arthur et al., 2004) and activation (Fukuyama et al., 2006).

But *Vav1/2/3<sup>-/-</sup>* BMM appear to have normal adhesion (Ch. 5.8), and spread normally in response to CSF-1 (Ch. 4.5). This suggests that the initial defect is in cell protrusion and not adhesion formation. And that this leads to reduced adhesion formation when cells fail to make protrusions.

Signal transduction during the spreading of Wt and *Vav1/2/3<sup>-/-</sup>* BMM was compared by following tyrosine phosphorylation of cellular proteins, as well as the modification of paxillin. In *Vav1/2/3<sup>-/-</sup>* BMM tyrosine phosphorylation and the phosphorylation of paxillin were not potentiated during spreading as in Wt BMM. Thus it appeared that the spreading defect in the absence of Vav proteins was being reflected in reduced signalling in response to adhesion. Spreading cells make cell protrusions upon initial adhesion to the surface. These are stabilised by new adhesions that lead to further phosphorylation of signalling proteins such as FAK, Src and the scaffold protein paxillin.

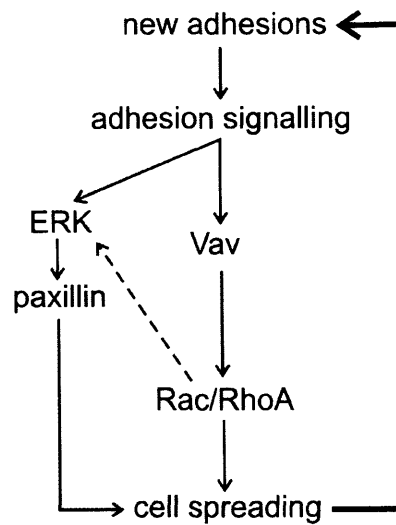
ERK1/2 signalling pathways are involved in the regulation of cell morphology and migration (Huang et al., 2004), and ERK activation downstream of integrins is well established (Hood and Cheresch, 2002; Giancotti and Ruoslahti, 1999). It was observed that ERK1/2 phosphorylation during spreading was also much lower in *Vav1/2/3<sup>-/-</sup>* BMM. ERK1/2 could influence cell spreading through the phosphorylation of paxillin, which was phosphorylated less in *Vav1/2/3<sup>-/-</sup>* BMM during spreading. Paxillin can be



phosphorylated directly by ERK. Bellis and colleagues reported that in addition to phosphorylation on tyrosine residues, paxillin was phosphorylated on serine residues in response to cell adhesion on fibronectin (Bellis et al., 1997). It has been shown that phosphorylation on Ser126 and Ser130 was dependent on the Raf-MEK-ERK signalling cascade (Woodrow et al., 2003), and phosphorylation on serine residues of paxillin was required for cell spreading (Richardson et al., 1997). Thus, paxillin phosphorylation during spreading could, in part, be catalysed by ERK1/2. Proper phosphorylation of paxillin could be essential in the formation of integrin signalling complexes. Thus, reduced activation of ERK1/2 in *Vav1/2/3<sup>-/-</sup>* BMM could also be a cause of defective spreading.

Signalling through the ERK1/2 pathway could regulate spreading through cross-talk with Rho GTPases. The serine/threonine kinase PAK1 associates with Rac1 upon cell interaction with the ECM (del Pozo et al., 2000). Introduction of activated forms of Rac, Cdc42 or RhoA into cells induces an activation of ERK2 in the presence of activated Raf (Frost et al., 1996). More detailed study showed that PAK1-catalysed phosphorylation of MEK1 was a possible mechanism for this activation (Coles and Shaw, 2002). In addition, Eblen and colleagues showed an adhesion-dependent association between MEK1 and ERK, which could be inhibited by a dominant-negative form of PAK1 (Eblen et al., 2004). Though these findings were based mainly upon co-expression and over-expression studies, they do highlight a possible mechanism of interaction between Rho GTPase and MAPK signalling pathways. Thus, the observed reduction in ERK1/2 activation during spreading of *Vav1/2/3<sup>-/-</sup>* BMM could be a consequence of the absence of Vav GEF activity on Rac.

However, there is also evidence that places ERK1/2-mediated paxillin phosphorylation upstream of the activation of FAK and Rac (Ishibe et al., 2004). This demonstrates the complications of studying a process that could be regulated in the initial stages by a positive feedback mechanism (Figure 5.11).



**Figure 5.11: Model for adhesion-dependent signalling in BMM.**

Though integrins can mediate cell adhesion, other cell surface receptors also affect cell adhesion and spreading. It was demonstrated that integrins are not essential for cell adhesion, though they are required for spreading in platelets (Goodman et al., 1993). The Syndecans are a family of cell-surface proteoglycans that are able to bind a variety of extracellular ligands (see review (Carey, 1997)). Though syndecans signal to many intracellular processes, their main role appears to be to modulate the adhesion formation by the integrins (Bass et al., 2007; Wilcox-Adelman et al., 2002). It would therefore be interesting to determine which surface proteins regulate adhesion-dependent spreading of macrophages on glass and plastic.

In summary, it appears that Vav family of proteins have critical but redundant functions in macrophage signalling in response to adhesion. Vav proteins are required for optimal activation of Rac1 and RhoA upon BMM adhesion. ERK1/2 and paxillin phosphorylation is lower following adhesion of macrophages that lack the Vav proteins. This could be an indirect consequence of reduced spreading, or further effects of the reduced signalling through RhoA or Rac1. Further study is needed to determine the hierarchy of signalling events involving Vav proteins, Rho GTPases, ERK1/2 and paxillin during macrophage spreading.

## **6. Concluding Remarks**

In this study it was found that macrophages lacking all three isoforms of Vav had defects in morphological regulation and signalling in response to adhesion. Furthermore these macrophages had an elongated morphology in culture, and enhanced migratory persistence. However, Vav proteins were not required for macrophage chemotaxis or morphological and signalling responses to CSF-1.

None of the altered properties of macrophages lacking all three isoforms of Vav was due to the loss of any single isoform, apart from slightly reduced migration speed in the absence of Vav1. This may be a consequence of the experimental approach: the role of the Vav proteins in macrophage migration and morphological regulation was investigated by analysis of macrophages derived from Vav-deficient mice. All experimental systems have advantages and disadvantages. For example, many studies into the function of this family of proteins have utilised constitutively active or dominant negative mutant forms of Vav. These studies have given valuable insights into the potential role of Vav proteins in leukocyte functions. However, introduction of these proteins into cells could interfere with the function of other endogenous proteins non-specifically. The gene targeting approach avoids non-specific interference with other proteins but the data generated must still be treated with caution. When a gene is expressed within a cell it occupies a particular site or sites according to its binding motifs. Gene knock-out studies attempt to determine the function of a protein as implied by the consequences of its absence from the cell/organism. However, if a binding site is vacant it may become occupied by another protein that is capable of recognising it. Thus, knock-out cells can exhibit phenotypes as a result of the absence of the protein combined with the results of compensation by other related proteins.

This could be why the absence of single Vav isoforms of Vav did not result in any deficiency or alteration in the aspects of macrophage functions tested in the study. The Vav proteins may therefore have redundant functions in regulating macrophage morphology and migration, but similarly, other Rac-

GEFs may be able to replace certain Vav functions when all three are absent. To resolve these issues, it would be useful to down regulate Vav isoforms transiently and specifically, for example by the use of small interfering RNA (reviewed by (Novina and Sharp, 2004)). siRNA expression in cells specifically interferes with the level of proteins that are produced from mRNAs that contain the identical sequence. Attempts to introduce exogenous DNA into primary macrophages were unsuccessful, however some groups have successfully introduced DNA constructs into primary macrophages using retroviral vector systems (Vedham et al., 2005). This approach has been used to investigate the role of Vav1 in the phagocytosis of human bacterial pathogens (Schmitter et al., 2007). Alternatively, a conditional knock-out approach could be adopted, as was used by Wells and colleagues to study the role of Rac1 in macrophages (Wells et al., 2003). Interestingly, a method has been developed to study the loss of function of an endogenous protein, where it is still expressed in the cell. The so-called 'knock-in' approach introduces a loss-of-function mutation into the gene sequence that still allows expression of the protein. This method was used to study the isoform-specific function of the p110 $\alpha$  subunit of PI3K in mice (Foukas et al., 2006). Mutations could be introduced into the Vav DH domain, to investigate isoform-specific GEF-dependent activities of Vav proteins.

BMM lacking all three Vav proteins adopted an elongated morphology in culture. However, when the cells were induced to migrate more efficiently by seeding on bacterial plastic this elongation was still present though it was more transient. Cell elongation also correlated with increased migratory persistence. The Rho GTPases are critical for regulation of the actin cytoskeleton and adhesion during migration. Rac is thought to regulate cell protrusion and RhoA is required for retraction. Interestingly, Rac1 and Rac1/2 deficient macrophages can still migrate (Wells et al., 2003), and inhibition of the RhoA/ROCK pathway modifies migration but does not abolish it (Smith et al., 2003). Thus, these cellular components regulate cell migration without any one of them being absolutely required for it in vitro. Similarly, Vav proteins are not required for migration per se, but are certainly required to maintain normal migration in vitro. The next step in the study therefore, would

be to investigate the *in vivo* consequences of the altered migratory properties of macrophages lacking Vav proteins.

It has been demonstrated that cells in a three dimensional matrix adopt a very different morphology than that which is observed in two-dimensional culture (Martins and Kolega, 2006). In culture fibroblasts adopt a flat morphology, whereas in a fibronectin matrix, they adopt an elongated morphology (Yamada et al., 2003). Furthermore, the dynamics of cell protrusion and retraction during migration also appear to be different in a 3-D environment (Knight et al., 2000). It is possible that alterations in migratory properties *in vitro* could have more significant effects on the migration of these cells in 3-D.

Another aspect of cell migration *in vivo* is matrix degradation/remodelling. In culture, there is no requirement for matrix degradation to achieve cell translocation, whereas *in vivo* it may be essential. This is a process where zinc-dependent endopeptidases (termed matrix metalloproteinases, MMPs) catalyse the degradation of ECM components. These enzymes are either secreted, or expressed on the cell surface. Matrix degradation and its role in cell migration has been studied mainly in the context of tumour cell invasiveness. From studies in tumour cell lines it has become clear that the Rho GTPases are involved in the regulation of MMPs. Rac1 was shown to be required for collagen-induced MMP-2 activation in fibrosarcoma cell lines (Zhuge and Xu, 2001), and RhoA and Rac1 were required for SDF-1-induced MT1-MMP up-regulation in melanoma cells (Bartolome et al., 2004). Furthermore, the SDF-1-stimulated upregulation of MT1-MMP expression was inhibited by siRNA knock-down of Vav1 and Vav2 (Bartolome et al., 2006). Macrophages are known to express a subset of MMPs (Trask et al., 2001). Considering the evidence suggesting the involvement of Rho GTPases in the regulation of MMPs, it would be interesting to study if Vav proteins contribute to this regulation in macrophages.

Leukocytes are exposed to a number of different chemical stimuli when they are recruited to sites of inflammation or infection. *In vitro*, macrophages are known to chemotax to such stimuli as C5a (Richards et al., 1983), MCP-1 (Jones et al., 2003) and RANTES (Weiss-Haljiti et al., 2004). In this study, it

was found that Vav proteins are not required for macrophage responses to CSF-1. However, it may be that Vav proteins are specifically required for chemotaxis to other macrophage chemo-attractants.

Leukocyte sensing of directional inflammatory stimuli is a factor that directs their recruitment and migration to the site of inflammation. However, a preceding step is the firm adhesion of the leukocyte to the endothelium, after which cells extravasate into the underlying matrix. In this study it was found that macrophages lacking Vav proteins had a reduced ability to spread upon adhesion to glass and plastic. Firm adhesion to the endothelium is largely mediated by leukocyte integrin receptors binding endothelial cell-expressed ICAMs (reviewed in (Alon and Feigelson, 2002)). Adhesion to glass and plastic is also largely dependent on integrins. This defect in adhesion-dependent spreading *in vitro* may therefore suggest a role for Vav proteins in regulating the transition from an adhered state to that in which cells can migrate through the endothelium, via regulation of cell adhesions and cell morphology. Though many studies have reported a role for Vav in integrin-mediated processes in leukocytes, how this affects the ability of these cells to traverse the endothelium has not been described. It would therefore be important to study the role of Vav proteins in macrophage transmigration through an endothelial layer. Intravital microscopy is a technique whereby the transendothelial migration of leukocytes can be viewed by the imaging of blood vessels of live, anaesthetised animals (Yadav et al., 2003). Thus, the rolling, adhesion and extravasation events can all be observed in real-time, and could be compared between Wt and Vav-deficient BMM populations.

It was clear that Vav1/2/3<sup>-/-</sup> BMM had defects in morphological changes that occur upon adhesion. However, the adhesion of Wt and Vav1/2/3<sup>-/-</sup> BMM was not directly compared. There are a number of ways in which adhesion can be measured. Most involve plating the cells in suspension into a well or dish and then quantifying the number of adherent cells after a specific time. In these assays, quantification of cell numbers is complicated by the existence of the fluid meniscus in the plated cell suspension, which results in an uneven cell density of adherent cells across the surface. Counting of cells can be avoided

if adhered cells are measured by spectrophotometry or fluorescence, which is usually achieved by adding a dye to the live cells, although the dye could affect the behaviour of the cells and their adhesive properties. In addition, these assays cannot discriminate between varying strength or stages of adhesion. It was clear that Vav1/2/3<sup>-/-</sup> BMM had no defect in the ability to adhere, but there may have been differences in the strength, integrity or kinetics of adhesion formation that could not have been measured in a consistent way by static adhesion assays. These factors can be investigated when adhering cells are exposed to a consistent force. For example cells in suspension can be perfused through an observable channel. The rate at which cells roll and eventually arrest on the substrate can be taken as a measure of their adhesive properties. Since the flow rate can be fixed, cells are adhering against a constant force. This method has previously been used to study the adhesion of platelets to collagen (Pearce et al., 2004), and the adhesion of neutrophils to the  $\beta 2$  integrin ligand, C3bi (Gakidis et al., 2004). Such an assay would also more closely approximate the physiological conditions in which leukocytes become adherent when they are recruited from the circulation. In the blood vessel, leukocytes interact with the endothelium under fluid flow, and as they roll on the endothelial layer, contacts are made and broken before the cell finally becomes firmly adherent (see review, Alon and Feigelson, 2002). Thus, the study of the role of Vav proteins in macrophage migration and adhesion can be progressed by studying these properties in more physiologically relevant contexts.

The product of proper adhesion and migration is that the macrophage can be efficiently recruited to the site of inflammation, and the requirement of Vav proteins therein, can be studied by measuring macrophage localisation to specific sites within the organism in response to inflammation. For example, inflammation can be stimulated by injection of a pro-inflammatory stimulus into an air pouch made under the skin. Several hours after the injection, the number of leukocytes that have infiltrated the region can be quantified. This method has previously been used to investigate the role of PYK2 in macrophage migration (Okigaki et al., 2003), and PI3Ky in inflammation (Hirsch et al., 2000).

Leukocyte recruitment to sites of inflammation is a key step during inflammatory or immune responses. Once at the inflammatory focus, macrophages are required for clearance of pathogens by phagocytosis, microbial killing by release of superoxide, potentiating the inflammatory response by the release of pro-inflammatory cytokines and antigen presentation. Interestingly the Rho family of GTPases are also involved in the regulation of these aspects of leukocyte function. The Rho GTPases RhoA, Rac and Cdc42 are required for the regulation of the actin cytoskeleton during the process of phagocytosis (Caron et al., 1998; Yamauchi et al., 2004). Vav proteins have also been implicated in this process. Vav1 and Vav3 have been shown to be required for complement-mediated phagocytosis in both neutrophils (Gakidis et al., 2004) and macrophages (Hall et al., 2006). Vav proteins can regulate the morphological changes associated with phagocytosis in macrophages, and therefore could be important for pathogen clearance at inflammatory sites.

Leukocyte production of superoxide acts to kill microbial pathogens. It has been established that the Rho GTPase Rac controls cellular production of superoxide by binding to the NADPH oxidase complex (Nishimoto et al., 1997). In addition, Rac2-deficient macrophages have defects in superoxide production in response to FcγR stimulation (Yamauchi et al., 2004). Transient expression of a number of Rac GEFs in COS cells expressing the NADPH oxidase complex revealed that constitutively active Vav1 induced the highest superoxide production, even though other GEFs induced greater Rac activation (Price et al., 2002). Furthermore, the p67<sup>phox</sup> component of the NADPH complex was shown to recruit Vav1, thereby effecting positive feedback regulation of superoxide production (Ming et al., 2007). It has also been reported that Vav1 is required for fMLP-induced superoxide production in neutrophils (Kim et al., 2003).

Thus, the involvement of Vav proteins in regulating leukocyte function is not limited to migration and adhesion. Moreover, Vav proteins also regulate other Rho GTPase-dependent processes independent of cytoskeletal reorganisation.



To conclude, it is clear from this study and others that Vav proteins are essential signalling components linking extracellular stimuli to intracellular signalling and cytoskeletal regulation. Using a gene knock-out approach it has been shown that Vav proteins are not required for macrophage responses to the chemo-attractant and growth factor, CSF-1. However, Vav proteins do have critical but redundant functions in regulating macrophage migration and adhesion-dependent morphological changes. Further study should reveal how these functions are required for macrophages in vivo, and ultimately how Vav protein function affects the innate immune responses of the organism.

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